Surviving contrasting abiotic stress

An ecophysiological study on responses to flooding and drought stress in *Solanum dulcamara*

Qian Zhang

ISBN: 978-90-825123-6-6

Cover design: Onno W. Calf
Photos: Heidrun Huber and Qian Zhang
Layout & Printed by: proefschrift-aio.nl

The research presented in this thesis was performed at Department of Experimental Plant Ecology, Institute for Water and Wetland Research, Faculty of Science, Radboud University Nijmegen, the Netherlands. Qian Zhang was supported by a grant from China Scholarship Council.

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Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op woensdag 21 september 2016
om 14.30 uur precies

door

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Doctoral Thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken,
according to the decision of the Council of Deans
to be defended in public on Wednesday, 21st September 2016
at 14:30 precisely

by

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Contents

Chapter 1

General introduction

Flooding and drought stress

Water is an important resource directly or indirectly shaping the natural patterns of plant distribution and biodiversity (Silvertown *et al.* 1999; Silvertown, Araya & Gowing 2015). Excessive (flooding) and insufficient (drought) water availability can both impose stress to terrestrial plants occurring in natural but also in agricultural systems, often resulting in declines in biodiversity and crop production (Jackson & Colmer 2005; Perata, Armstrong & Voesenek 2011; Farooq *et al.* 2012). Due to global climate change, the occurrence of drought and excessive rainfall are predicted to increase in frequency and intensity in the near future (IPCC 2013). The resulting shift in flooding and drought patterns will strongly affect plant growth, thus challenging future breeding programme and the ability to predict the implications for natural vegetation patterns and community structures. Unravelling the mechanisms of how tolerant plant species cope with these contrasting water related stress factors will provide valuable insights for crop breeding and management of natural plant ecosystems under the commonly accepted scenario predicting increased frequency and intensity of flooding and drought events.

Flooding and drought lie on the opposite ends of the hydrological gradient. They impose completely different stresses to plant growth. During flooding, low oxygen stress is considered to be a major problem for flooded plants (Voesenek *et al.* 2004), because the gas exchange rates between plants and their environment are severely reduced due to the 10 000 times slower gas diffusion in water than in air (Armstrong 1979; Jackson 1985). The available oxygen is easily depleted in the rhizosphere through the high oxygen demand by respiration of plant roots and microorganisms in the soil (Zitomer & Lowry 1992; Sauter 2013). This oxygen limitation then impairs aerobic respiration, ultimately causing energy deficiency for plant development and functionality (Bailey-Serres & Voesenek 2008). Additionally, decreased nutrient availability in the flooded soil and accumulated toxic compounds produced by the anaerobic metabolism of plant roots and microorganisms (Pucciariello *et al.* 2014), impose further negative effects on plant growth (Končalová 1990; Blom & Voesenek 1996).

Drought stress, on the other hand, hampers plant growth by limiting water uptake. The imbalance between water loss and water uptake leads to water deficiency in plant tissues, which results in reduced tissue water content, diminished leaf water potential, closed stomata and loss of turgor (Jaleel *et al.* 2009). Water deficiency in the cells causes damage to the integrity of the membrane and constrains cell expansion and division (Mahajan & Tuteja

2005; Jaleel *et al.* 2009), thus leading to decreased growth rates and biomass accumulation (Farooq *et al.* 2012). Drought also inhibits plant growth through reducing nutrient mobility in the soil, thereby reducing nutrient uptake (Farooq *et al.* 2012). Severe drought stress ultimately dehydrates the entire plant and causes plant death (Bray 1997).

As apparent from the distinctly different negative effects of drought and flooding on plant performance, it is evident that different sets of adaptive traits must have evolved enabling plants to cope with these contrasting stresses. The different sets of traits seem to make it unlikely for a single plant species to perform well at both extremes, which is also reflected in the distinctly different community composition at both ends of the moisture gradient. Yet, some plant species still manage to occur along the entire gradient. Here the question arises which mechanisms these species deploy that enable them to deal with such contrasting stress factors.

Mechanisms for plants to cope with contrasting stress

It is generally accepted that two major mechanisms exist enabling plants to occupy contrasting environmental conditions, local adaptation and adaptive plasticity. One way to survive contrasting environments is by evolving different ecotypes, each adapted to its local habitat, which is known as 'local adaptation'(Savolainen, Lascoux & Merilä 2013). In contrasting habitats, selection pressures including abiotic and biotic factors differ markedly, thereby potentially resulting in opposing traits being selected for in the same species subjected to these different environmental conditions (Kawecki & Ebert 2004; Savolainen, Lascoux & Merilä 2013). As a result, under a given set of environmental conditions, local genotypes can have a higher fitness than genotypes from foreign habitats (Sambatti & Rice 2006; Wright, Stanton & Scherson 2006). Thereby local adaptation allows a plant species to thrive in spatially variable environments (Becker *et al.* 2006; Ellis, Weis & Gaut 2007; von Wettberg, Remington & Schmitt 2008).

An alternative mechanism enabling plant species to occur over a wide environmental gradient or in contrasting environmental conditions is the ability to express high levels of adaptive phenotypic plasticity (Agrawal 2001; Kawecki & Ebert 2004). Adaptive phenotypic plasticity allows a genotype to produce several different phenotypes, each favoured by its local environment without the necessity of genetic differentiation (Pigliucci, Murren & Schlichting 2006). Instead of evolving locally adapted populations to gain higher fitness in local habitats, phenotypic plasticity enables plants to express adaptive plastic traits

to sustain a relatively high fitness across habitats. Phenotypic plasticity has been predicted to evolve under small grained environmental heterogeneity, where conditions can change throughout the life-span of a given organism or across generations (Nicotra et al 2010; Murren et al 2015)

Whether evolution favours local adaptation or phenotypic plasticity depends on a variety of factors, such as the species, the traits under selection, strength of the selection pressures, gene flow, spatial grain and frequency of temporal fluctuations and costs and constraints associated with phenotypic plasticity. To investigate which of the two strategies has evolved in a single species occurring in contrasting hydrological habitats, one needs to compare both the genetic structure and phenotypic traits of plants from the contrasting habitats with a decent number of replicates (Kawecki & Ebert 2004). It is then important to also understand which plant traits contribute to surviving a stress factor, in other words, which mechanism does the plant possess to withstand the stress?

Plant acclimations to flooding stress

As oxygen deficiency is the major constraint during flooding, maintaining sufficient internal aeration is key for flooding resistance (Sauter 2013). A very important acclimation that facilitates internal oxygen transport is aerenchyma, a porous tissue type that is constitutively present or induced upon flooding inside plant shoots and roots (Voesenek & Bailey-Serres 2015). This aerenchyma tissue connects the submerged plant parts with the non-flooded parts, allowing oxygen diffusion from the atmosphere to the submerged plant organs (Voesenek & Bailey-Serres 2015). As sediment roots of terrestrial plants usually are thin and have a relatively low porosity, these roots are easily prone to oxygen shortage, impairing root growth and functioning and ultimately plant survival (Armstrong *et al.* 1991). To maintain a functioning root system during flooding, many wetland plant species develop a superficial adventitious root system to replace or support the sediment roots (Sauter 2013). These adventitious roots contain large proportions of aerenchyma to promote oxygen diffusion throughout the root (Thomson *et al.* 1990; Visser *et al.* 2000). In some species, a lateral diffusion barrier is established in adventitious roots to minimize radial oxygen loss (Colmer 2003). Also, adventitious roots have been shown to take up oxygen from the floodwater, which further contributes to plant internal aeration (Ayi *et al.* 2016). With enhanced internal aeration, adventitious roots are hypothesised to take over the function of the primary roots in terms of water and nutrient uptake, thus leading to improved plant performance during flooding (Sauter 2013).

However, the atmospheric contact cannot always be maintained or restored (Voesenek *et al.* 2006). For instance, in deep flooding, the floodwater surface might be too high for the plants to protrude, resulting in complete submergence of the entire plant. This may be particularly common for floodplain species at young developmental stages. To cope with such complete submergence, several plant species are able to extend their shoots to above the water surface by enhanced shoot elongation to restore the essential contact with the atmosphere, thus escaping from the completely submerged conditions. Other species, rather than investing into escape acclimations, rapidly slow down metabolism to reduce oxygen and energy consumption and use the carbon storage to fuel longer survival under prolonged deep flooding (Perata & Alpi 1993; Crawford & Braendle 1996; Bailey-Serres, Lee & Brinton 2012). In contrast to the "escapers", plants deploying such a quiescence strategy stop growing until the subsidence of the water level (Voesenek & Bailey-Serres 2013).

It is likely that a specific strategy and/or acclimation is only employed by plants under specific ecological settings. Apart from the dependence of different survival strategies on flooding types, the expression of adaptive changes leading to flooding acclimation may also be constrained by complete submergence. In complete submergence, gas exchange is further impeded, causing the efficiency of respiration and photosynthesis to be largely reduced (Setter & Laureles 1996; Manzur *et al.* 2009). Light as another primary resource for plant growth is also substantially reduced in deep water, restraining photosynthesis to even lower levels (Mommer & Visser 2005). Under such aggravated stress conditions, plants may fail to generate sufficient energy to deploy high-energy demanding plastic responses. As a consequence, complete submergence may constrain the plastic morphological acclimation plants have evolved to cope with shallow partial flooding. Moreover, expression of the plastic acclimations may also be influenced by the age of the flooded plants (van der Sman, Blom & Barendse 1993). Since the timing of flooding is often not predictable, plants can be exposed to flooding stress at any time in their life cycle (van der Sman, Blom & Barendse 1993). As indicated above, plants at young developmental stages are more prone to be fully submerged compared to those at more mature stages (Nabben, Blom & Voesenek 1999). Consequently, this raises the question whether plants are able to deploy the same acclimations in response to partial flooding and complete submergence, and whether plants at young developmental stages require different adaptations promoting survival than adult plants.

Plastic acclimations clearly confer fitness advantages to the flooded plants (Visser, Blom & Voesenek 1996; Chen *et al.* 2011). However, these plastic responses require resources and thereby may incur fitness costs to plant growth if the investment cannot be compensated by the benefits (Murren *et al.* 2015). The balance between benefits and costs associated with a specific plastic response to flooding may depend on the flooding regime, especially the duration of flooding. The investment into plastic responses may take a long time to be paid back, therefore potentially causing fitness decline when flooding lasts for short periods of time.

Plant acclimations to drought stress

Lack of water is a very different type of stress than flooding, requiring specific adaptations. Drought tolerant species have evolved a set of traits enabling them to cope with water deficiency by reducing water loss and/or improving water uptake (Farooq *et al.* 2012). Most water loss of the plant is controlled by the stomata, rapid regulation of stomatal closure is therefore an important trait for drought tolerance (Jaleel *et al.* 2009). Traits that reduce light absorbance and thus photosynthesis, such as rolling leaves to minimize area for light absorbance, can also effectively reduce plant water loss (Ehleringer & Cooper 1992). Similarly, decreased canopy leaf area by reduced growth and shedding older leaves, and reduced specific leaf area (SLA) by changing leaf structures substantially contribute to water saving during drought events (Chaves, Maroco & Pereira 2003). In addition, increased water use efficiency is also important for plants to maintain the water balance under mild water deficiency (Chaves, Maroco & Pereira 2003). Nevertheless, acclimations that allow conservative water use and reduced water loss may only be helpful for plants to withstand transient water shortage with soil water availability being regained afterwards. Under prolonged drought stress, maintaining sufficient water uptake is crucial for plant growth and survival.

Enhanced water uptake under drought conditions is primarily achieved by a variety of adaptive root traits. Extensive root systems that increase resource exploration are thought to be beneficial for water extraction from the drying soil (Comas *et al.* 2013). Many drought tolerant plant species are capable of rapidly adjusting the patterns of biomass allocation upon drought stress (Poorter *et al.* 2012). Relatively more investment into the root system enhances water absorbance from the soil (Jackson, Sperry & Dawson 2000). Furthermore, root architecture and root distribution along the vertical axis also determine the spatial root exploration in the soil, thus potentially resulting in

differences in water uptake (Lynch 1995; Manschadi, Christopher & Hammer 2006). Since drought is a progressive stress, when the topsoil dries out, the deep soil layers may still be moist. Therefore, sending roots to the moist deep soil is essential for maintaining water uptake during prolonged drought events (Gowda *et al.* 2011). This deep rooting trait that potentially enhances water capture from deep sources is common in arid regions and is generally recognized as an essential feature for drought tolerance, thus becoming a subject in plant breeding efforts aiming to increase drought tolerance in crop species (Manschadi *et al.* 2010; Lynch & Wojciechowski 2015).

The maximum rooting depth varies significantly among species and among genotypes within a species (Manschadi *et al.* 2010; Wasson *et al.* 2014), but also differs in the same genotype growing in different environments (Wasson *et al.* 2014). A deep root system might be a result of fast root elongation, which may be favoured by drought conditions (Wasson *et al.* 2012). However, under moist conditions, nutrient acquisition may require a shallow rooting system (Ho *et al.* 2005). Several nutrients, especially phosphorous, distribute superficially near the soil surface with decreased availability along soil depth (Jaleel *et al.* 2009), thus potentially requiring a contrasting root system phenotype (shallow rooting) than the ideal drought phenotype (deep rooting). As a result, deep rooting might be differentially expressed in plants that are under different selection regimes.

Overall, flooding and drought stress select for completely different adaptations, often resulting in a different set of species occurring along a flooding-drought gradient. It is therefore of great value that individual species do exist which can cover the whole gradient. These species form an ideal model system to study adaptation to contrasting stress and preferable over studying the adaptation of different species, as in the latter case it may be difficult to distinguish specific adaptations from evolutionary constraints.

Aim of this thesis

The main goal of this thesis is to investigate the mechanism for plants to survive flooding and drought stress. To study this, I investigated the ecophysiological responses of plants to both flooding and drought stress in a wild perennial species *Solanum dulcamara* (Bittersweet; Solanaceae family). This species is characterised by a wide spectrum of ecological niches, ranging from permanently flooded wetlands to extremely dry coastal dunes, and from shaded under-story habitats to open exposed areas. It is therefore an ideal candidate to study plant adaptation to contrasting stress. *S. dulcamara* is also

a close relative to many crop species in the *Solanaceae* family, such as potato, tomato, eggplant and pepper. The knowledge gained in this species may lead to better insights into improving crop tolerance to flooding and drought stress.

I first investigated in **Chapter 2** whether local adaptation occurred in plants originating from contrasting habitats in *S. dulcamara*. Seeds were collected from a wet (the border of dune slacks) and a dry (top of sand dunes) habitat from each of nine locations along the coastline of the Netherlands. Plants from seeds of these 18 populations were used to assess the genetic and phenotypic differentiation between the two contrasting habitats. The phenotypic responses between habitats were compared under control, flooding and drought conditions.

In **Chapter 3**, I addressed the question whether plants at young developmental stages employed different survival strategies in response to partial and complete submergence than mature plants. I specifically investigated the flooding-induced adventitious root formation and plant performance in juvenile and adult plants under partial and complete submergence using two out of the nine population pairs.

I investigated whether adventitious root formation conferred fitness benefits under partial flooding by experimentally manipulating the formation of these roots in **Chapter 4**. Since adventitious root formation requires energy consumption, potential costs may impose negative effects on plant performance if the costs cannot be compensated by the associated benefits. Whether the positive effects of adventitious roots outweigh the associated costs of root production may be time dependent. Therefore, I also investigated whether duration of flooding influences the relative benefits of adventitious roots, and linked this relationship to the functioning of adventitious roots.
Complete submergence is a compound stress that may induce changes in a variety of associated factors which in turn may affect the potential of plants to produce adventitious roots. Therefore, in **Chapter 5**, I investigated the factors potentially influencing adventitious root formation in complete submergence by altering oxygen supply, plant carbohydrate status, and by supplying extra auxin.

Roots were confined to relatively shallow soil layers in Chapter 2 which limited the ability of plants to elongate the roots to capture moisture in deeper soil layers. As a result the plants may not have been able to utilize the whole suite of traits evolved to increase drought tolerance. Local adaptation might have evolved in traits such as deep rooting or changed root architecture. In **Chapter 6**, I investigated in three population pairs population differentiation in root traits in both, a laboratory screening of early rooting architecture

conducted on germination papers and a greenhouse drought experiment in later developmental stages conducted in deep tubes allowing roots to reach deep soil layers to potentially benefit from higher water supply in those deeper soil layers.

In concert the results revealed that despite the genetic differentiation, there was no evidence of local adaptation shaping flooding and drought tolerance in *S. dulcamara*, but high levels of plasticity in essential traits. Flooding tolerance was mainly determined by adventitious roots which essentially contributed to resource uptake, but the benefits of which were only apparent under long flooding duration. High levels of plasticity may have been favored by negligible costs for maintenance and expression of plastic responses.

Chapter 2

Hydrologically contrasting environments induce genetic but not phenotypic differentiation in *Solanum dulcamara*

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Published in Journal of Ecology (2016). DOI: 10.1111/1365-2745.12648 (in press)

Abstract

In contrasting habitats, locally adapted populations are expected to evolve through directional selection. Hydrological gradients provide a scenario where strong selection forces have led to species segregation in communities along the hydrological niche axes due to specific adaptations to their ambient environment. However, much less is known about the processes enabling species with wide ecological amplitudes, such as *Solanum dulcamara* L., to occupy the contrasting niches at the far ends of the gradient. Here we investigate the question whether local adaptation is indeed the driving mechanism enabling this species to inhabit contrasting wet and dry habitats. Using molecular analyses and performing a common garden experiment, we examined whether genetic and phenotypic differentiation had occurred between populations originating from flooding- and drought-prone habitat pairs from nine different locations. We also tested whether plants were better adapted to their home site stress. We found significant genetic variation but hardly any phenotypic differentiation between populations from contrasting habitats for plants grown under optimal conditions. Surprisingly, comparison of the genetic differentiation in neutral markers with that in phenotypic traits using the Q_{ST} - F_{ST} (estimated by Φ_{PT}) approach revealed that the Q_{ST} values were consistently and significantly smaller than the Φ_{PT} values. This is in contrast to the hypothesis that directional selection is an important component enabling *S. dulcamara* to survive under contrasting habitat conditions. In addition, no home site effects were detected for responses to flooding and drought treatments. All plants were characterized by high levels of plasticity for traits associated with flooding- and drought-tolerance, such as rapid adventitious root formation upon flooding and increased root to shoot ratio in response to drought, irrespective of habitat of origin. Moreover, no trade-offs in such flooding and drought responses were found. Our results show that *S. dulcamara* has not evolved locally adapted populations in response to flooding and drought stress, despite genetic differentiation and despite the presumably strong selection gradient. The generally high levels of adaptive plasticity in traits increasing flooding- and drought- tolerance may be the main mechanism allowing *S. dulcamara* to occupy hydrologically contrasting habitats.

Introduction

Directional selection is commonly believed to be the driving force leading to locally adapted genotypes or ecotypes, thereby enabling species to inhabit contrasting habitat types (Hall & Willis 2006; Sambatti & Rice 2006; Wright, Stanton & Scherson 2006; Stroup 2015). However, results from empirical studies conducted on species occurring in such divergent habitats do not always match this prediction (Callahan & Pigliucci 2002; Chen *et al.* 2009; Gimeno *et al.* 2009; Anderson & Geber 2010; Souther, Lechowicz & McGraw 2012). This indicates that the evolution of locally adapted genotypes may depend on the specific ecological settings as well as on other constraints, such as lack of genetic variation and a high frequency of gene flow (Galloway & Fenster 2000; Anderson & Geber 2010). Adaptive phenotypic plasticity, which enables a genotype to produce multiple phenotypes each adapted to its local environment, has been proposed as an alternative strategy enabling plants to cope with short-term changes in environmental conditions (Pigliucci, Murren & Schlichting 2006; Pratt & Mooney 2013). With habitat conditions becoming less predictable due to increasing climate extremes, the latter mechanism of adaptation may lead to improved performance under these changing conditions as compared to the relatively slow selection processes leading to the evolution of new ecotypes (van Gerven *et al.* 2015). However, such plasticity would typically not be the main mechanism expected in more stable environments.

Hydrology is one of the major factors shaping the vegetation patterns and is expected to be altered by climate change (Silvertown *et al.* 1999; Fraaije *et al.* 2015). Spatial niche segregation has been widely found along hydrological gradients (Silvertown, Araya & Gowing 2015). Flooding and drought, as the two contrasting extremes of the hydrological gradient, impose completely different constraints for growth to plant species, *i.e.* oxygen deficiency *vs.* water shortage (Bailey-Serres & Voesenek 2008; Jaleel *et al.* 2009). Consequently, different phenotypic traits are expected to be selected for by these two stress factors. We investigated for a widespread plant species that occupies both extremes of a hydrological gradient whether ecotype formation explains its wide ecological amplitude, by measuring the genetic variation and morphological responses of populations sampled from the field, in a common garden experiment.

Traits including enhanced shoot elongation, adventitious root development and aerenchyma formation are selected for in flooding-resistant plant species (Visser, Blom & Voesenek 1996; Colmer & Voesenek 2009), whereas traits such as strong regulation of stomatal closure, large and deep root systems and

high water use efficiency are favoured in drought-tolerant species (Jaleel *et al.* 2009). Some of these traits which increase flooding- and drought-tolerance are mutually exclusive. For instance, drought favours deep rooting capacity (Wasson *et al.* 2012; Lynch 2013), whereas in flooded conditions plants usually grow their roots very shallowly in the upper soil layer where oxygen might still be available (Baker *et al.* 2001). These contrasting phenotypes in response to the two stress factors indicate that divergent selection for these traits is very likely to occur, reminiscent of the distribution of species with different flooding adaptations over flooding gradients (Lenssen *et al.* 2004; van Eck *et al.* 2004; Voesenek *et al.* 2004). However, contrary to this expectation, our previous work showed little difference in the expression of phenotypic traits and performance between populations of *Solanum dulcamara* L. originating from flooding- and drought-prone habitats of two independent locations when subjected to partial flooding (Visser *et al.* 2015). Therefore, the question arises if ecotype formation is indeed a mechanism that explains the wide distribution of this species across distinctly different habitats. A comparison of a larger number of contrasting habitat pairs is needed to answer this question, as relying on data from a small number of locations carries the risk that local conditions and random genetic effects may conceal general patterns.

Bittersweet (*Solanum dulcamara*) is a perennial species which occurs in a broad hydrological range of habitats, from continuously flooded conditions to dry conditions with soil water content reaching levels as low as 3 % (w:w; unpublished data, Q. Zhang). We conducted a common garden experiment in a greenhouse with nine population pairs, each representing one wet and one dry habitat collected in the same geographical area. We examined both the genetic and phenotypic differentiation as well as plastic responses to the environment, to investigate the mechanisms enabling *S. dulcamara* to occur in both continuously flooded as well as in drought-prone environments. We specifically addressed the following questions: (1) Does genetic variation exist between populations originating from wet and dry habitats? (2) Do consistent phenotypic differences occur between these local populations when subjected to optimal, common garden conditions? (3) Do populations from wet and dry habitats differ in their responses to the environmental stress factors flooding and drought? (4) If so, do these local populations show an improved performance when subjected to their home site stress?

Material and methods

Species and seed collection

Solanum dulcamara is a perennial Eurasian species within the Solanaceae family. It is outcrossing (Golas *et al.* 2010) but self-compatible (Wright & Barrett 2010), and also capable of vegetative regeneration (Waggy 2009). *S. dulcamara* occurs in a broad range of ecologically contrasting habitats, ranging from permanent wetlands to dry costal dunes (Dawood *et al.* 2014; Visser *et al.* 2015).

In autumn 2012 and 2013, seeds of *S. dulcamara* were collected at nine locations along the coast of the Netherlands (Fig. 1). At each location, habitats with contrasting hydrological characteristics were present. A wet population was sampled at the shore of freshwater dune lakes where plants are typically partially submerged throughout the year. A dry population was sampled at dry primary sand dunes well above the seasonally flooded dune slacks, where plants are extremely unlikely to experience flooding. For the dry habitats, the top soil dries out completely in summer, e.g., in the dry dune habitat in Texel soil moisture content reached values of around 2 ± 0.7 % (w:w; n=3) at the soil surface and 3 ± 0.6 % (n=2) in 45-50 cm deep soil in late spring (Q. Zhang, unpublished results). At each habitat, we collected seeds from as many different individual plants as possible (at least ten to 15 individuals)

Figure 1. Schematic overview of the nine sampling locations along the coast of the Netherlands. At each location, two contrasting habitats, *i.e.*, freshwater dune lakes and dry primary sand dunes, are present at, on average, less than two kilometres apart.

with a minimum distance of 5 m between plants to get a representative seed sample covering the genetic variation present in each population. Depending on the habitat of origin, individual plants experienced either only flooding or only drought stress throughout their life-time. The distance between wet and dry habitats from a given location was 2 km on average and ranged from 0.7 to 4.7 km, which is within the maximum pollination and/or seed dispersal distance (Pasquet *et al.* 2008), leading to a high potential of gene flow between these two habitats in each location. The minimum distance between neighbouring locations was 13.8 km. After collection, seeds of each population were pooled, cleaned, dried at room temperature and then stored at 4 °C.

Plant material

A hundred and fifty seeds from each of the 18 populations were surface sterilized with 15 % (w:v) trisodium phosphate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) solution for 20 minutes and then rinsed carefully with tap water. After sterilization, seeds were sown in seed trays with cells of 3.5 × 3.5 × 4 cm (length × width × depth) filled with moist commercial sowing compost (117.6 mg L^{-1} N, 58.8 mg L^{-1} P, 125.2 mg L^{-1} K, 4.2 mg L^{-1} Mg, 159.6 mg L^{-1} S, 0.25 mg L^{-1} B, 1 mg L^{-1} Cu, 0.76 mg L^{-1} Fe, 1.3 mg L^{-1} Mn, and 0.3 mg L^{-1} Zn; Horticoop substrate, Lentse potgrond & Slingerland potgrond, Cuijk, the Netherlands), covered by plastic and subsequently kept at 4 °C in a cold chamber for three weeks to break the dormancy. Thereafter, seed trays were transferred to the greenhouse for germination for three weeks. Seed trays were covered with transparent plastic for two weeks to maintain high humidity and stimulate germination. Temperature and humidity in the greenhouse were on average 17.5 ± 0.3 ˚C (mean ± SD) and 65.5 ± 5.2 % (mean ± SD), respectively, during the day, and 15.6 ± 0.1 ˚C (mean ± SD) and 65.1 ± 5.1 % (mean ± SD), respectively, during the night. Homogeneously germinated seedlings were transplanted into individual pots of 1.35 L, containing nutrient-poor soil (2.77 mg L^{-1} NO_3^-, 0.05 mg L^{-1} NH_4^+, and 0.06 mg L^{-1} PO_4^{3-}; 70 % sand and 30 % clay; v:v) supplied with 4 g L^{-1} slow release fertilizer (Osmocote® Exact Standard, NPK 15-9-12+2 MgO + tracing elements, release time 5 - 6 months, Everris International B.V., Geldermalsen, the Netherlands). Each plant received 60 ml of nutrient solution (2 g L^{-1} Kristalon in tapwater, Yara International ASA, Vlaardingen, the Netherlands) one week after transplanting to avoid nutrient limitation at the onset of the experiment. Plants were regularly watered in the greenhouse for five weeks before the onset of the treatments.

DNA isolation and AFLP analysis

Amplified fragment length polymorphism (AFLP) markers were used because of their advantages for assessing genetic differentiation among populations (Mueller & Wolfenbarger 1999; Gaudeul *et al.* 2004). Twenty-four plants from each of the 18 populations were selected to perform the AFLP analysis. To isolate DNA, the CTAB DNA miniprep (Weigel & Glazebrook 2002) was modified as described below. A young leaf of about 2 cm^2 was collected from each plant, frozen in liquid nitrogen and ground in a Mixer Mill MM300 (Retsch GmbH, Haan, Germany) for 30 s at 30 Hz. The frozen leaf tissue was suspended in 500 µl CTAB buffer (0.1 M Tris-HCl pH 7.5, 0.7 M NaCl, 0.01 M EDTA pH 8 and 1 % CTAB (w:v)) and incubated at 60 °C for 40 min. A chloroform-isoamylalcohol extraction was performed by adding 250 µl chloroform-isoamylalcohol (24:1, v:v), mixing well and centrifuging for 4 min at 151 g. Two hundred microliters of the watery upper phase was added to new collection tubes containing the same volume of isopropanol. After mixing, 10 min incubation at room temperature and centrifugation at 151 g for 10 min, the pellet was recovered, washed with 70 % ethanol (v:v) and vacuum dried. The DNA pellet was then dissolved in 200 µl MilliQ water.

The AFLP analysis was performed as described by Peters *et al.* (2001). Three different primer combinations were used: [1] *Eco*RI+AAC (GACTGCGTACCAATTC**AAC**) + *Mse*I+CTG (GATGAGTCCTGAGTAA**CTG**), [2] *Eco*RI+ACC (GACTGCGTACCAATTC**ACC**) + *Mse*I+CAA (GATGAGTCCTGAGT AA**CAA**) and [3] *Eco*RI+ACC (GACTGCGTACCAATTC**AAC**) + *Mse*I+CTT (GATGAGTCCTGAGTAA**CTT**).

Common garden experiment

Treatments

For each of the 18 populations, 27 plants were used for the greenhouse experiment. These 27 plants from each population were equally and randomly assigned to control (well-watered), flooding or drought treatments, resulting in nine replicates for each treatment. Plants subjected to flooding treatment were flooded to 10 cm above soil surface in nine square containers (length x width x height, 98 x 98 x 28 cm) each containing one plant from each of the 18 populations. The soil moisture for control plants and drought-treated plants was kept at 25 % and 12 % (v:v), respectively, by replenishing the lost amount of water every 1-2 days. The volumetric water content in the soil was measured with a theta probe (HH2, Moisture Meter version 2, Delta-T Devices LTD, Cambridge, UK). Wilting occurs in this soil-pot system when soil moisture is

below 10 % (Q. Zhang, personal observation). The treatments lasted for three weeks, after which the plants were harvested. This duration of the experiment was chosen based on the rapid morphological responses of this species to different environmental factors in previous studies, where the consequences of the morphological changes were observed within two to three weeks (Visser *et al.* 2015; Q. Zhang unpublished data).

Measurements

Five extra plants from each population were harvested one day before the onset of the treatments to determine initial plant height (68.0 ± 1.4 cm, n = 18 populations) and biomass (2.92 ± 0.08 g, n = 18 populations). The youngest fully expanded leaf on the main shoot from each plant was marked with a permanent marker pen one week after the onset of the treatments, to indicate which leaves fully developed during the treatments. One day before the final harvest, the leaf above the marked leaf was scanned with a scanner (Epson Expression 11000XL, Japan) and the size of the leaf was determined by the projected area calculated with WinRHIZO (Regent Instruments Inc., Québec, Canada). At the final harvest, adventitious roots were counted and harvested separately. The height of the main shoot, number of side branches, and the length and thickness of the internode above the marked leaf were determined. Internode thickness was measured using a digital vernier calliper. Plants were also scored for the presence of flowers at harvest time. No fruit was set at that time. Root systems were carefully washed, and plants were then divided into roots, stems and leaves and dried to constant weight at 70 ˚C, after which dry weights were measured.

Data analyses

AFLP data

Fragment scoring was performed manually from the gel images and absence and presence of bands were scored as 0 and 1, respectively. If a band could not be scored unequivocally, a missing value was indicated as -1. For each of the 18 populations, 20-24 individuals were scored and the three AFLP primer combinations resulted in 95 polymorphic AFLP loci: 28 from *Eco*RI+AAC/*Mse*I+CTG, 25 from *Eco*RI+ACC/*Mse*I+CAA and 42 from *Eco*RI+ACC/*Mse*I+CTT. The resulting binary matrix was analysed by a principal coordinate analysis (PCoA) in GenAlEx 6.5 (Peakall & Smouse 2012). A two-way ANOVA (type III sum of squares) was conducted on the first coordinate to test for the effects of habitat and location and their interaction (R Development Core Team 2014) using the *car* package (Fox & Weisberg 2011). Two separate nonparametric

Kruskal-Wallis tests were conducted due to non-homogeneous variance on the second coordinate to test for the effects of habitat and location, p values were adjusted according to the Bonferroni correction. Since some locations seemed to show specific wet and dry habitat clustering, an additional principal coordinate analysis was performed for each location separately. The derived first and second coordinates were then analysed by separate nonparametric Wilcoxon Mann-Whitney tests due to non-homogeneous variance to test whether these coordinates differed between habitats within each of the nine locations (Dytham 2011; Ravenscroft, Fridley & Grime 2014) in R. Bonferroni correction was conducted to adjust p values to take multiple comparisons into account. To determine the genetic differentiation between populations, pairwise population differentiation Φ_{PT} values were computed from analysis of molecular variance (AMOVA) using 999 permutations. A Mantel test, as implemented in GenAlEx 6.5, was performed using genetic and geographic distances to test for isolation by distance (Mantel 1967).

Phenotypic data

All analyses were conducted in R using the *lme4* (Bates *et al.* 2015) and *car* packages. Data were analysed using a split-plot analysis of variance (ANOVA, type III sum of squares) to test the main effects of treatment and habitat and their interaction. The split-plot model was built according to Crawley (2005). In this model, habitat was nested within the random factor location, and the habitat effect was tested over the mean sum of square of the "habitat × location" interaction error. The main effects and their interaction on the presence of flowers were analysed by conducting a logistic regression. Since branch number was count data, the main effects of treatment and habitat and their interaction on this variable were analysed by conducting a poisson regression. Separate one-way ANOVAs, logistic regressions and poisson regressions comparing plants from dry and wet habitats under each treatment were then performed to determine the direction of the interaction between treatment and habitat on root to shoot ratio, root biomass, branch number and the presence of flowers. The habitat effect on number and biomass of adventitious roots were analysed with one-way ANOVA (type III sum of squares) where habitat was treated as main effect and location as random effect. Total root biomass was reciprocally transformed, and leaf size was transformed by a power transformation where the constant *e* was raised to the power of the leaf size data, and the other data were log10 transformed if necessary to increase the homogeneity of the variances.

To test whether a consistent phenotypic difference occurred between habitats and among populations under common garden conditions, a principal component analysis (PCA) using the built-in *prcomp* function was performed for all nine locations. A subset of the traits was used for the analyses, including total biomass and seven phenotypic traits, *i.e.* root to shoot ratio, stem height, internode length, stem thickness, branch number, leaf size and specific leaf area. Total biomass was included in the PCA analyses as a substitute of growth rate since the initial total biomass was similar across populations. Care was taken to avoid highly correlated or interdependent traits, e.g., we did not include root or shoot biomass separately, because we already had included total biomass and root to shoot ratio in the analyses. We performed a two-way ANOVA (type III sum of squares) on the PCA scores of both component 1 and 2 to test the overall effects of habitat and location and their interaction. As there was a significant interaction between habitat and location, and also because we were interested in the habitat difference in each location, the same PCA analysis was also performed for each location. A student's *t*-test was then conducted on the PCA scores of component 1 and 2 between dry and wet habitat within each location (Dytham 2011; Ravenscroft, Fridley & Grime 2014). To test whether a different general response to stress conditions existed between habitats over the nine locations, another PCA analysis was conducted on the relative stress responses of the populations calculated from the total biomass and the same seven phenotypic traits used in the separate PCA analyses. The response to stress including both flooding response and drought response was calculated as $100 \times (Mean_{flooding/drought}\text{-}Mean_{control})/Mean_{control}$, a positive or a negative value indicates an increased or decreased trait value in response to stress, respectively. To test the main effects of habitat and treatment and their interaction, a two-way ANOVA (type III sum of squares) was conducted on the PCA scores of component 1 and 2. As there was a marginally significant interaction between treatment and habitat and also due to our interest in the habitat effect under each treatment, separate PCA analyses for each treatment were conducted and their PCA scores of component 1 and 2 were compared, respectively, between wet and dry habitat for each treatment using separate student's *t*-tests (Dytham 2011; Ravenscroft, Fridley & Grime 2014). Bonferroni correction was conducted to adjust p values to take multiple tests into account for all the separate tests for the nine locations and three treatments.

Q_{ST} - F_{ST} comparison

A Q_{ST} - F_{ST} comparison was performed to detect local adaptation and to disentangle the effects of directional selection from that of other evolutionary

processes, such as genetic drift. F_{ST} is a measure of genetic differentiation among populations estimated by neutral molecular markers (Whitlock 2008). We used Φ_{PT} to estimate F_{ST} as Φ_{PT} is an analogue and a good estimator of F_{ST} (Steinger *et al.* 2002; Gruenthal, Acheson & Burton 2007; Le Roux *et al.* 2014). Φ_{PT} was calculated by subjecting the AFLP data to an AMOVA analysis in GenAlEx 6.5 as described in the section "AFLP data" of data analyses. For Q_{ST}, we first estimated the within- and between-habitat variance in total biomass and the other seven phenotypic traits used in the PCA analysis in a random effect ANOVA model using *lme4* package in R (R Development Core Team 2014), where habitat was treated as random effect (Steinger *et al.* 2002). These within- and between-habitat variances in total biomass and quantitative traits were then used to calculate Q_{ST}, which is a measure of the genetic differentiation among populations for quantitative traits (Whitlock 2008), with the equation $Q_{ST}=\sigma^2_{GB} / (\sigma^2_{GB} + 2\sigma^2_{GW})$, where σ^2_{GB} is the between-habitat variance, and σ^2_{GW} is the within-habitat variance (Leinonen *et al.* 2013). The average Q_{ST} for total biomass and the other seven phenotypic traits were compared with the Φ_{PT} for AFLP markers for all the nine locations using a paired student's *t*-test in R (R Development Core Team 2014).

Results

Genetic differentiation was present between habitats

Populations originating from different habitats and locations differed significantly in both the first and second PCoA coordinates, and a significant interaction between habitat and location existed as well (Appendix Table S1). Among the nine locations, the three North Sea islands (Schiermonnikoog, Ameland and Texel) together with Callantsoog and Castricum were separated from the other, more southern locations (Fig. S1, Table S1 & S2).

Within each location, the first principal component coordinate of plants originating from dry and wet habitats differed significantly (Fig. 2, Table S1). Among those eight locations which were characterized by significant differences, Schiermonnikoog, Zandvoort and Callantsoog were the most genetically separated population pairs according to their relatively high ΦPT values (0.288-0.394; Fig. 2), whereas the population pairs in Ameland and Goeree were the least separated (Φ_{PT} values 0.124-0.142; Fig. 2). The second principal component coordinates of plants originating from dry and wet habitats did not differ for any of the nine population pairs (Fig. 2). To investigate whether the different levels of genetic variation could be explained by the geographical distance among populations, a Mantel test was performed

between the genetic distance and the geographical distance for dry and wet populations within locations. Within location, a greater geographical distance between the two populations did not translate into a larger genetic distance (Fig. 3a), which was in contrast to the clear effect of geographical distance on molecular population differentiation at the larger, *i.e.*, location, scale (Fig. 3b).

Flooding and drought stress had significant effects on plant growth and phenotype

Flooding and drought significantly suppressed plant growth in terms of reduced biomass accumulation compared to well-watered control conditions (Table 1, Fig. 4a). Plants also displayed different traits in response to drought and flooding, resulting in phenotypes with higher root to shoot ratio under drought conditions and adventitious root formation in flooded plants (Fig. 4b, k, l). In addition, plants grew shorter and produced thinner main stems under stress than when growing under control conditions (Table 1, Fig. 4d, f). Flooding also significantly decreased specific leaf area (Table 1, Fig. 4i).

Plants from dry and wet habitats did not differ in response to treatments

Plants originating from dry and wet habitats showed some minor differences in phenotypic traits, such as root biomass, root to shoot ratio and branch number (Fig. 4, Table 1). Across the three treatments, plants originating from dry habitats had significantly higher root biomass and root to shoot ratio, and produced more side branches than those from wet habitats (Fig. 4, Table 1). Under control conditions, plants originating from dry habitats tended to invest more into root growth than those from wet habitats (Fig. 4c). When flooded, plants originating from dry habitats had shorter internodes than those from wet habitats (Fig. 4g). Habitat had no effect on flowering ratio of plants under control conditions; however, under flooding and drought conditions, plants originating from dry habitats had a higher flowering ratio than plants from wet habitats (Fig. 4j). This is due to different responses in flowering time of plants to the local and foreign stress. Plants from dry habitats did not respond to the foreign flooding stress but responded to their local drought stress with earlier flowering. Plants from wet habitats, on the contrary, did not respond to the foreign drought stress but delayed flowering when flooded (Fig. 4j). Remarkably, plants originating from either wet or dry habitats responded similarly, rather than differently, to the flooding and drought treatments for most morphological traits (Table 1, Fig. 4).

Figure 2. Diagrams presenting the main axes of a principal coordinate analysis (PCoA) on the genetic distance matrixes of plants originating from dry (open symbols) and wet (shaded symbols) habitats within each of the nine locations along the coast of the Netherlands, based on 95 AFLP markers. Significant results of the nonparametric Wilcoxon Mann-Whitney tests comparing the PCoA scores (Coord. 1; no significant results for Coord 2) between habitats per location are indicated with asterisk (**). The significance levels are adjusted into *** $p < 0.0001$, ** $0.0001 < p < 0.001$, and * $p < 0.006$ to take multiple comparisons into account. Each symbol represents an individual plant.

Consistent phenotypic differences existed between habitats under common garden conditions rather than under stress conditions

To test whether consistent phenotypic differences over a range of phenotypic traits existed between habitats under common garden conditions, a principal component analysis (PCA) was performed on total biomass and seven phenotypic traits (root to shoot ratio, stem height, internode length, stem thickness, branch number, leaf size and specific leaf area) of plants in the control treatment. The two-way ANOVA analysis on the PCA scores showed that location had a significant effect and habitat a marginally significant effect on the first component (Table S3). Neither habitat nor location affected the second component (Table S3). Separate comparisons of the PCA scores for each population pair revealed that for none of the nine population pairs, habitats were significantly separated along the first or second axis based on the phenotypic data (Fig. 5), with only marginally significant effects of habitat for Schiermonnikoog along the second axis and a marginally significant separation along the first axis for Meijendel (Fig. 5). Interestingly, only Schiermonnikoog was characterized by strong molecular differentiation, whereas Meijendel was the least separated population pair according to the AFLP data (Fig. 2). When the genetic differentiation for total biomass and quantitative traits (Q_{ST}) was compared with that for the AFLP markers (Φ_{PT}) between the wet and dry habitats within each location, we found significantly and consistently smaller values of Q_{ST} (range: 0.012 - 0.104; Table S2) than those of Φ_{PT} (range: 0.063 - 0.394; Table S4) ($t = -3.69$, $p = 0.006$). In addition to the lack of substantial differences for trait values under control conditions, habitat of origin did not affect the response of those traits to drought or flooding either (Fig. 6, Table S5).

Discussion

In line with our hypothesis that strongly contrasting habitats would lead to segregating populations, clear genetic differentiation occurred between populations of *Solanum dulcamara* originating from wet and dry habitats, indicating that there was a potential for divergent selection. Surprisingly, however, hardly any phenotypic differentiation among plants from different habitats occurred under common garden conditions. These results indicate that flooding and drought stress did not impose consistent directional selection pressures across locations and traits. We therefore did not find evidence for local adaptation explaining the wide ecological soil moisture range *S. dulcamara* can cover, which was also supported by the relatively lower between-habitat differentiation in terms of phenotypic (Q_{ST}) as compared to molecular (Φ_{PT})

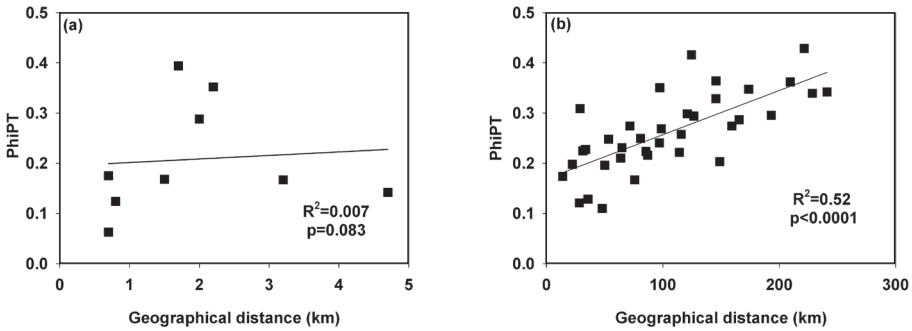

Figure 3. Results from a Mantel test analysing the relationship between the geographical distance and the genetic differentiation (Φ_{PT} (PhiPT)) (A) of plants between the wet and dry habitats of each location and (B) between any two out of the nine locations. The R^2 (proportion of explained variability) and the significance of the correlation are displayed in each graph.

Table 1. The F-values and their significance analysed by means of split plot ANOVA for the main effect of treatment, habitat and their interactions on total biomass, root biomass, root to shoot ratio, SLA, stem height, internode length and thickness of the internode, and Chi-squares and their significance of logistic regression on flowering ratio and poisson regression on branch number for the effect of treatment and habitat, and their interaction. Habitat effect was tested over the mean sum of square of the "habitat × location" interaction. Adventitious roots were included in root biomass and the calculation of root to shoot ratio. The significance levels are indicated as: *** p ≤ 0.001, ** p ≤ 0.01, * p ≤ 0.05, $ 0.05 < p < 0.1, ns p > 0.1. Significant values (p ≤ 0.05) are in bold.

		F-values/Chi-squares			
	d.f.	**Total biomass**	**Root biomass**	**Root/shoot ratio**	**Leaf size**
Treatment (T)	2	**60.485*****	**32.290*****	**46.212*****	0.833^{ns}
Habitat (H)	1	1.017^{ns}	**4.512***	**6.904***	1.482^{ns}
T×H	2	1.209^{ns}	2.889$^{\$}$	1.740^{ns}	1.801^{ns}

Table 1. Continued

F-values/Chi-squares					
SLA	**Stem height**	**Branch number**	**Internode length**	**Stem thickness**	**Flowering ratio**
51.829***	**70.347*****	1.074^{ns}	**46.538*****	**58.152*****	**18.591*****
0.300^{ns}	1.921^{ns}	**6.847****	1.366^{ns}	0.015^{ns}	0.076^{ns}
0.810^{ns}	0.300^{ns}	1.031^{ns}	2.700$^{\$}$	0.676^{ns}	**6.756***

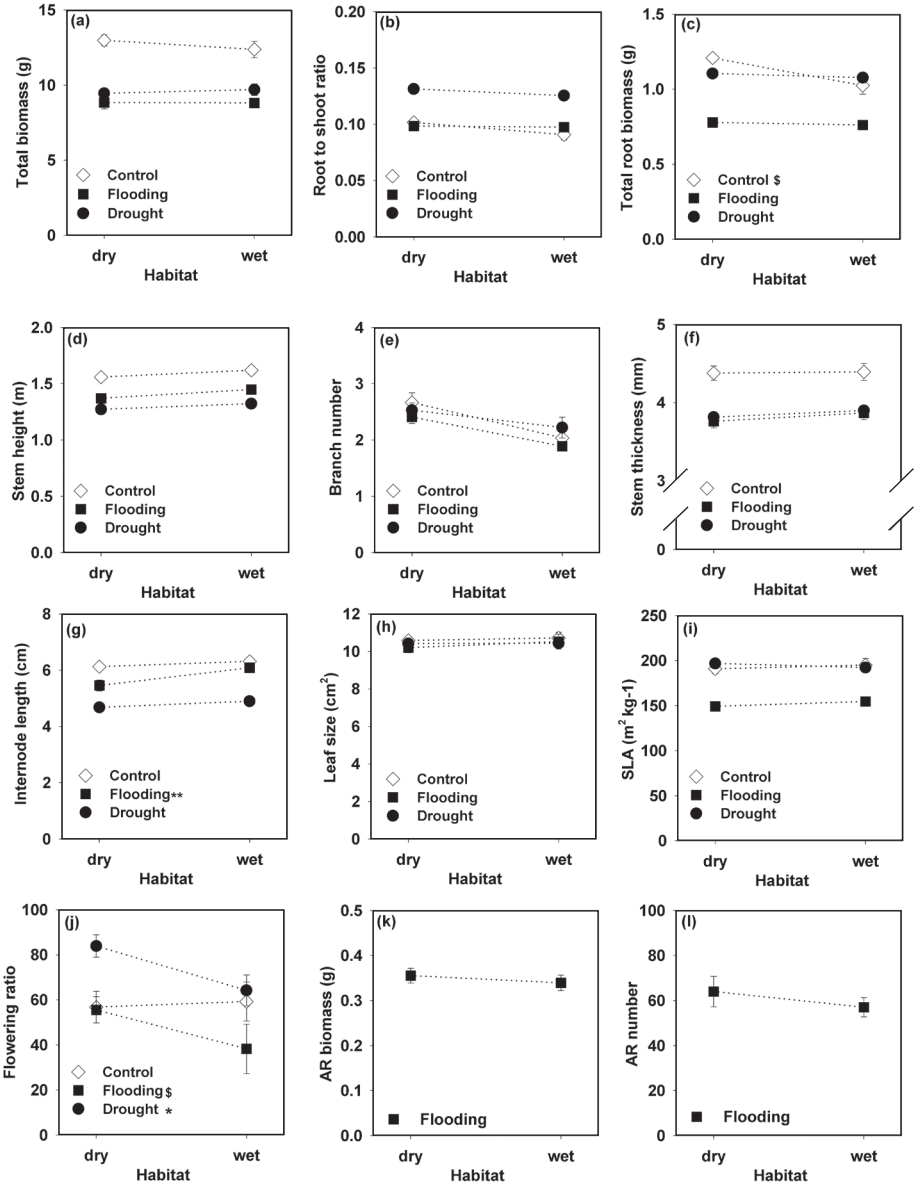

Figure 4. Phenotypic responses of morphological traits in populations originating from either dry or wet habitats in control, flooding and drought treatments. Adventitious roots were included in root biomass and the calculation of root to shoot ratio. Means are averages of nine populations; error bars indicate SE. (Marginally) significant differences between habitats in each treatment are indicated as: ** $p < 0.003$, * $0.003 < p < 0.017$, $ $0.017 < p < 0.03$, p values were adjusted according to the Bonferroni correction.

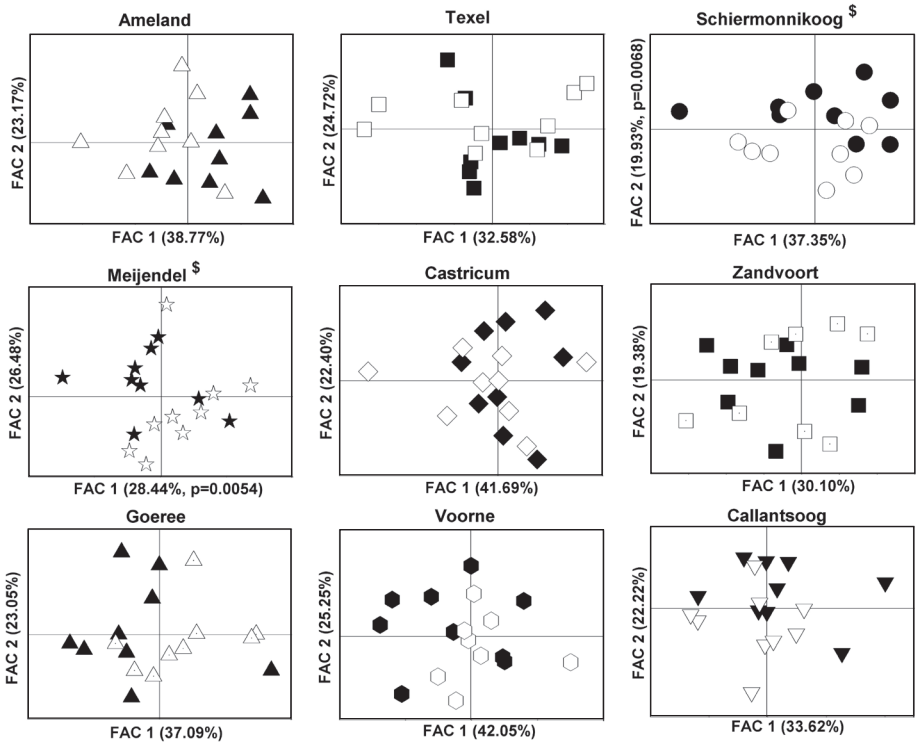

Figure 5. Diagrams presenting the main axes of a principal component analysis of plants originating from dry (open symbols) and wet (shaded symbols) habitats at nine locations along the coast of the Netherlands in control treatment. The PCA scores (Factor 1, 2) are derived from total biomass, root to shoot ratio, stem height, branch number, stem thickness, internode length, leaf size and SLA of individual plants. P values were adjusted according to the Bonferroni method, marginally significant results ($p < 0.01$) of the student's *t*-test comparing the PCA scores (Factor 1, 2) between habitats per location are indicated with p values in the X/Y axis and with $ in the title after the location name. Each symbol represents an individual plant.

values. In addition, the result $Q_{ST} < F_{ST}$ (estimated by Φ_{PT}) also suggests that the observed lack of differentiation in total biomass and phenotypic traits between habitats was not due to genetic drift ($Q_{ST} = F_{ST}$) (Leinonen *et al.* 2013). Moreover, no relative advantage in terms of increased performance was found in plants subjected to treatments reflecting their respective home site stress conditions, *i.e.*, flooding and drought. Apparently, despite the clear genetic differentiation, evolution of locally adapted populations in response to flooding and drought stress had not occurred in *S. dulcamara*.

Flooding and drought have not driven directional selection in *S. dulcamara*

Flooding and drought are strong selection forces that have been shown to affect distribution and abundance of plant species, leading to discriminating zonation patterns in natural habitats (Blom *et al.* 1994; van Eck *et al.* 2006; Silvertown, Araya & Gowing 2015). Locally adapted genotypes can be even selected for in species subjected to fine-grained spatial variation along flooding or drought gradients (Lenssen *et al.* 2004; Franks 2011). Therefore, more pronounced local differentiation may be expected when two divergent selection forces act on geographically separated (sub)populations of the same species (Heschel *et al.* 2002; Hereford 2009). *Solanum dulcamara* populations display very different phenotypes in their natural habitats when comparing plants growing in contrasting hydrological conditions (D'Agostino *et al.* 2013). In wet habitat plants are characterized by long, hardly brached stems and relatively large leaves while in dry habitat plants are characterized by short, highly branched stems and small leaves. In addition, locally adapted genotypes of this species have previously been revealed in response to light availability (Gauhl 1976; Gauhl 1979). We therefore expected to detect local adaptation in this species in response to water availability. However, *S. dulcamara* showed little evidence for adaptive population divergence in response to flooding and drought, despite the clear genetic differentiation between the two habitat types. Below we will explore the potential explanations for this result.

Lack of genetic variation and homogenizing processes such as gene flow have been argued to counteract adaptive natural selection (Galloway & Fenster 2000; Kawecki & Ebert 2004; Anderson & Geber 2010). However, the former reason does not apply to *S. dulcamara* in our study because of the relatively larger genetic variation as revealed by the AFLP measurements compared to the low differentiation of the phenotypic data. Moreover, despite the high potential for gene flow between different habitats through pollen and seed dispersal by bumblebees and birds, respectively, which are the main pollen and

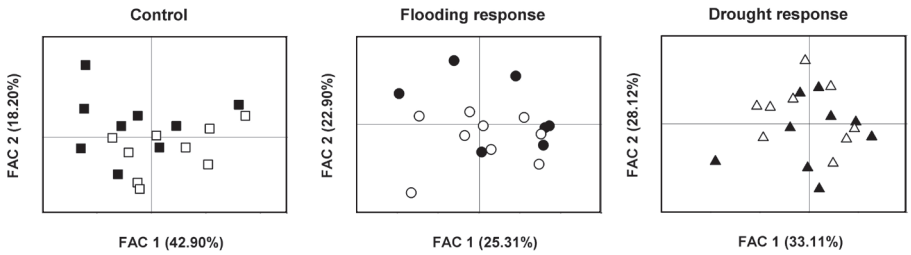

Figure 6. Principal component analysis of the plants originating from dry (open symbols) and wet (shaded symbols) habitats at nine locations along the coast of the Netherlands in flooding and drought treatments. The PCA scores (Factor 1, 2) for control are derived from the total biomass, root to shoot ratio, stem height, branch number, thickness of the stem, internode length, leaf size and SLA of a population. The PCA scores (Factor 1, 2) for flooding response and drought response are derived from the responses of the above eight variables to flooding and drought. Each symbol represents a dry or wet population from one location.

seed dispersal agents for this species (Pasquet *et al.* 2008; Waggy 2009; Golas *et al.* 2010), the observed genetic differentiation between populations within one location suggests gene flow among populations to be limited. Limited pollen flow may be explained by the composition of the local insect pollinator community, which can be very different due to the contrasting environmental conditions and plant species composition in the dry and wet habitats (Lobo, Quesada & Stoner 2005). If the activity range of these insect pollinators is limited to a specific local habitat, exchange of genes between habitats is not likely to occur, leading to genetic divergence. This provides a pre-requisite for directional selection to take place in the two contrasting habitats (Kawecki & Ebert 2004). Similarly, berries of *S. dulcamara* can be eaten and distributed by birds, but bird species frequenting marshes and dune lakes may not be the ones commonly found in the dry dunes. Therefore, a high level of gene flow does not seem a likely explanation for the apparent absence of locally adapted populations in *S. dulcamara*.

Local adaptation also depends on the strength of the divergent selection (Kawecki & Ebert 2004; Leimu & Fischer 2008). When the selection pressures are either mild or when intense temporal fluctuation exists, local adaptation is unlikely to occur (Kawecki & Ebert 2004; Leimu & Fischer 2008). In our study, however, there is no doubt that the flooding and drought stress acting on *S. dulcamara* in their local habitats is strong enough for divergent selection to occur, as in other species ecotype formation has occurred even along a much less pronounced hydrological gradient and at much smaller distances (Lenssen *et al.* 2004; Kooyers *et al.* 2015). Moreover, wet and dry *S. dulcamara* populations

co-occurred with a completely different set of species in their respective natural habitats (*i.e.*, marsh and reed vegetation, such as *Phragmites australis*, *Epilobium hirsutum* and *Lythrum salicaria*, *vs.* dry dune vegetation, such as *Ammophila arenaria, Oenothera spec.*, and *Hippophae rhamnoides*; personal observation). Seasonal variation might counteract local adaptation, and especially the frequency and severity of flooding may fluctuate. To minimise this potential effect, we collected seeds only from populations typically experiencing flooding throughout the year, located along the border of dune lakes that rarely dry up even at the end of summer. Therefore, environmental fluctuations or weak direct selection pressures are an unlikely driving force leading to the limited variation in response to wet and dry conditions among the plants from the two types of habitats.

Although difference in water availability was the most prominent environmental factor differentiating these habitats, we cannot rule out that other environmental factors have also played the role and have led to phenotypic changes that we have not measured. In addition, we did not include the reproductive stage in this experiment. Differences in flowering time in plants subjected to different conditions and different plasticity in flowering phenology in plants originating from different habitats may have contributed to local differentiation and may have led to genetic differentiation. In order to test this hypothesis a long term experiment, covering the reproductive stage of plants until seed set accompanied by additional measurements of phenology under natural conditions would be required.

Phenotypic plasticity may have replaced local adaptation

Our results clearly show that ecotypic differentiation for flooding and drought stress did not occur in our study, and populations from wet and dry habitats showed very similar plastic responses to flooding and drought. For instance, irrespective of habitat of origin, all plants produced numerous adventitious roots, a common flooding acclimation response facilitating gas exchange of flooded plants (Sauter 2013), thus allowing water and nutrient uptake to continue (Jackson 1955; Herzog *et al.* 2015). Adventitious roots have also been shown to positively correlate with plant biomass development of *S. dulcamara* in a previous study (Zhang *et al.* 2015). In this study, the fact that the adventitious rooting ability did not differ between populations from dry and wet habitats is quite surprising and suggests little differential selection among habitat types on this trait. Similarly, drought also induced changes in plant morphology, such as an increased relative investment in the root system, a response typically leading to increased water uptake potential (Gonzalez

et al. 2013). Lack of difference in such a trait between the two contrasting habitats reinforces the interpretation that there is no apparent trade-off in the ability of plants to adapt either to drought or to flooding. Interestingly, the difference in flowering time in response to flooding and drought suggests that local adaptation in this trait may result in modified plasticity rather than trait means. Altering flowering time in response to flooding and drought stress may result in different flowering phenology in the local plant populations. Such differences in phenology may well contribute to the genetic differentiation at small spatial scales found in our study.

This lack of differences in the expression of inducible flooding- and drought-adaptive traits between populations from contrasting habitats may explain why plants did not show an advantage in terms of lower reduction of performance if subjected to their home site stress. Apparently, all populations were able to maintain sufficient phenotypic plasticity to cope with both contrasting stresses, which raises the question why the ability to respond plastically to a foreign, thus hardly ever occurring, stress had not been selected against. This is in contrast to the well described trade-offs between flooding and drought tolerance among species (Araya *et al.* 2011; Silvertown, Araya & Gowing 2015). Possibly, the costs of maintaining this 'redundant' plasticity are not very high in this species (Weijschedé *et al.* 2006; Chen *et al.* 2011). Still, it is surprising that plants, irrespective of habitat, maintained equal level of plasticity in these traits, and irrespective of the opposite requirement on rooting pattern under flooded and drought conditions (shallow *vs.* deep rooting, respectively). This indicates that there is no developmentally fixed trade-off between flooding and drought induced rooting patterns, and that selection had apparently favoured high versatility in this trait above developmental fixation (Silvertown *et al.* 1999; Silvertown, Araya & Gowing 2015). The mechanism underlying this lack of trade-off in flooding and drought induced rooting patterns needs further investigation.

In line with our results, it has recently been argued that a high level of phenotypic plasticity is the major strategy enabling the marsh grass species *Spartina alterniflora* to spread over 19 ° latitude along the east coast of China across different soil moisture conditions, since the different phenotypes found in the field disappeared when plants were grown in a common garden (Liu *et al.* 2016). In our study, phenotypic plasticity even enables *S. dulcamara* to colonize habitats at the opposite ends of a soil moisture gradient. It remains an intriguing unsolved question why this species is able to cover such a wide ecological amplitude, without any apparent specific adaptation to the local environment, while most other species are much more confined to a smaller

range in the hydrological gradient (Voesenek *et al.* 2004; Moeslund *et al.* 2013). Further study of the characteristics that make *S. dulcamara* such a unique example has a potential to reveal mechanisms increasing our understanding on plant adaptation to different hydrological conditions.

Conclusions

Our results show that *Solanum dulcamara* has not evolved locally adapted populations in response to flooding and drought stress, despite the presumably strong selection gradient. Our results suggest that high levels of adaptive plasticity in the responsive traits in this species, regardless of habitat of origin, is an important mechanism allowing *S. dulcamara* to occur over a wide hydrological gradient. Such high levels of plasticity allowing plants to rapidly respond to environmental changes will most likely enable *S. dulcamara* to be resistant to and potentially even to expand its range in a changing climate. Our results also indicate that existing genetic differentiation does not necessarily predict differences in phenotypic responses to the environmental conditions, and vice versa.

Acknowledgements

We thank Fini De Gruyter and Loes Gubbels for performing the molecular experiment and Peter Cruijsen, Hannie de Caluwe, Annemiek Smit-Tiekstra, Xinping Yang, Simone Beljaars, Onno Calf, Janneke M. Ravenek, Gerard van der Weerden and co-workers at the Nijmegen Experimental Garden for their help in the common garden experiment. We also appreciate the advice on Q_{ST}-F_{ST} comparison from Philippine Vergeer and Rafael Rubio de Casas. We gratefully acknowledge the detailed and stimulating comments of two anonymous reviewers and the editor. This work is part of the B'sweet collaborative research initiative of the Departments of Experimental Plant Ecology and Molecular Plant Physiology of the Radboud University Nijmegen. Collection of seeds was supported by a grant from Stichting Schure-Beijerinck-Popping Fonds, and permission from Natuurmonumenten, Zuidhollands Landschap, Staatsbosbeheer and Waternet to sample populations at their properties. Qian Zhang was supported by China Scholarship Council grant number 201206510011. We declare no conflict of interests.

Data accessibility

Data from this paper can be accessed through the Dryad data repository (http://dx.doi.org/10.5061/dryad.4ht08) (Zhang et al. 2016).

Appendix

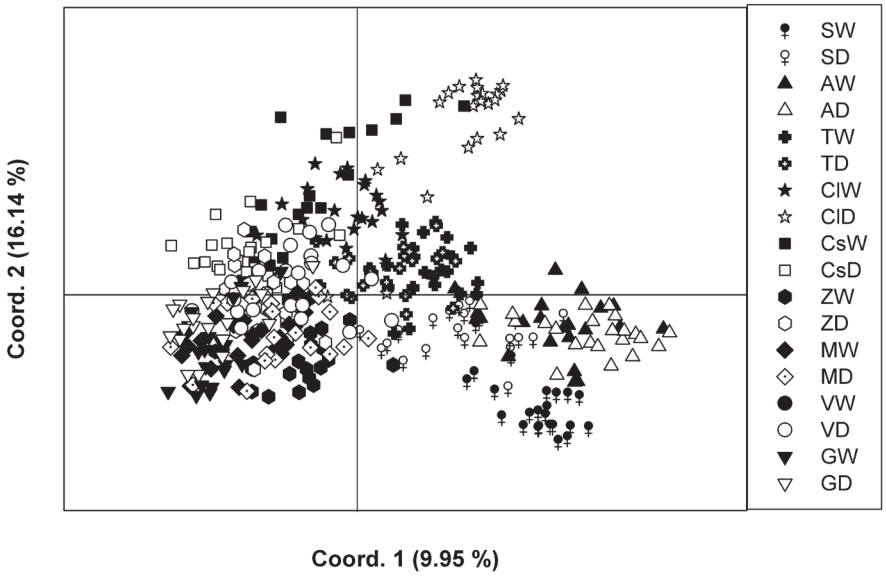

Figure S1. Results from a principal coordinate analysis (PCoA) based on the genetic distance matrix of all 18 *S. dulcamara* populations based on 95 AFLP markers. Each symbol type represents each of the nine locations, open symbols represent the dry habitat and closed symbols represent the wet habitat. S=Schiermonnikoog, T=Texel, Cl=Callantsoog, Cs=Castricum, Z=Zandvoort, V=Voorne, A=Ameland, M=Meijendel and G=Goeree. W=Wet (freshwater lake) habitat and D=Dry (dunes) habitat. Each point represents a plant individual, each population contains 20-24 plants.

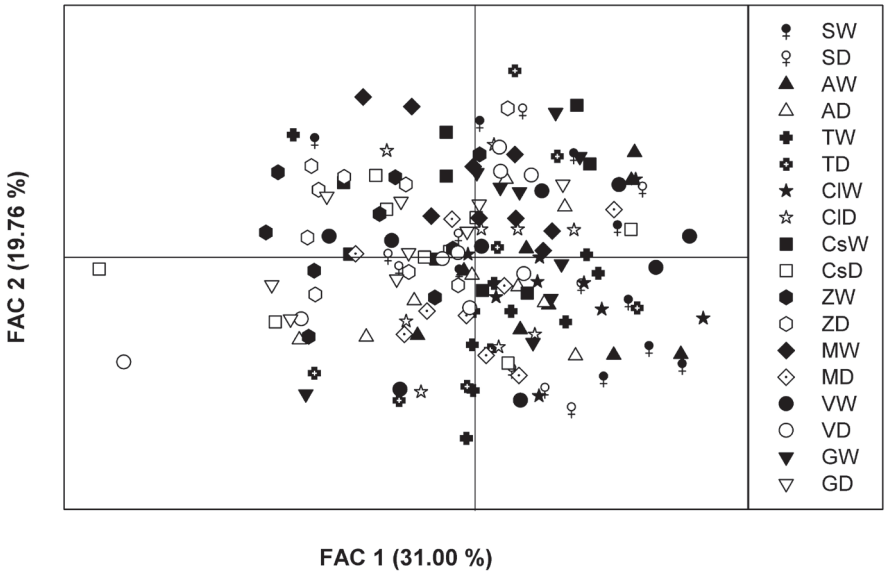

Figure S2. Diagrams presenting the main axes of a principal component analysis of plants originating from dry (open symbols) and wet (shaded symbols) habitats at nine locations along the coast of the Netherlands in control treatment. The PCA scores (Factor 1, 2) are derived from total biomass, root to shoot ratio, stem height, branch number, stem thickness, internode length, leaf size and SLA of individual plants. Each symbol type represents each of the 18 populations, each point represents an individual plant. S=Schiermonnikoog, T=Texel, Cl=Callantsoog, Cs=Castricum, Z=Zandvoort, V=Voorne, A=Ameland, M=Meijendel and G=Goeree. W=Wet (freshwater lake) habitat and D=Dry (dunes) habitat.

Table S1. The F-values and their significance analysed by means of two-way ANOVA for the effects of habitat and location and their interaction on the first coordinate (Coord. 1) generated from the PCoA analysis on all the nine population pairs, and Chi-squares and their significance of Kruskal-Wallis tests for the effects of habitat and location on the second coordinate (Coord. 2). P values were adjusted for the two comparisons on coordinate 2. The significance levels are indicated as: *** $p < 0.001$ (0.0005), ** $p < 0.01$ (0.005), * $p < 0.05$ (0.01), values inside the brackets are for coordinate 2. Significant values ($p < 0.05$ for coordinate 1 and $p < 0.01$ for coordinate 2) are in bold.

		F-values/Chi-squares	
	d.f.	**Coord. 1**	**Coord. 2**
Habitat (T)	1	**6.334***	**13.502*****
Location (L)	8	**179.236*****	**274.110*****
H×L	8	**19.094*****	N. A.

Table S2. The genetic differentiation Φ_{PT} values (below diagonal) and the geographical distance (above diagonal) between the 18 populations of *S. dulcamara*. The cells with thick borders represent the values between the two habitats of each location. S=Schiermonnikoog, T=Texel, Cl=Callantsoog, Cs=Castricum, Z=Zandvoort, V=Voorne, A=Ameland, M=Meijendel and G=Goeree. W=Wet (fresh lake) habitat and D=Dry (dunes) habitat.

SW	SD	AW	AD	TW	TD	ClW	ClD	CsW	CsD	ZW	ZD	MW	MD	VW	VD	GW	GD	
-	2.0	29.5	28.8	98.7	98.3	121.0	120.4	145.3	145.0	163.9	166.1	192.7	192.6	228.3	227.5	242.6	238.6	SW
0.288	-	29.0	28.3	98.9	98.5	121.6	121.0	146.2	145.9	164.8	167.1	193.7	193.6	229.4	228.5	243.6	239.6	SD
0.458	0.284	-	0.8	71.5	71.0	97.4	96.6	124.4	124.0	144.2	146.4	173.5	173.3	209.6	208.4	223.1	218.9	AW
0.506	0.327	0.124	-	72.3	71.8	98.2	97.4	125.3	124.8	145.0	147.2	174.3	174.1	210.4	209.2	223.9	219.7	AD
0.385	0.170	0.211	0.291	-	0.7	34.0	32.5	65.0	64.0	85.6	87.5	114.0	113.6	149.4	147.6	161.2	156.7	TW
0.538	0.350	0.382	0.448	0.175	-	34.7	33.2	65.7	64.7	86.3	88.3	114.7	114.4	150.1	148.3	161.9	157.4	TD
0.512	0.327	0.461	0.514	0.266	0.438	-	1.7	31.0	30.0	51.6	53.6	80.3	80.0	116.0	114.3	128.3	123.8	ClW
0.553	0.381	0.405	0.461	0.288	0.458	0.394	-	32.5	31.5	53.1	55.1	81.6	81.3	117.3	115.6	129.5	125.0	ClD
0.515	0.288	0.392	0.458	0.218	0.347	0.318	0.297	-	1.5	20.7	22.8	49.8	49.5	85.8	84.4	98.9	94.6	CsW
0.526	0.311	0.472	0.517	0.290	0.363	0.382	0.428	0.168	-	21.6	23.6	50.6	50.3	86.5	85.1	99.4	95.1	CsD
0.566	0.371	0.464	0.508	0.304	0.477	0.423	0.521	0.399	0.422	-	2.2	29.4	29.2	65.5	64.2	79.0	74.8	ZW
0.464	0.288	0.404	0.468	0.231	0.347	0.335	0.411	0.233	0.225	0.352	-	27.2	27.0	63.3	62.0	76.8	72.6	ZD
0.486	0.310	0.368	0.432	0.240	0.349	0.363	0.411	0.269	0.265	0.276	0.208	-	0.7	36.1	34.9	49.9	45.9	MW
0.421	0.221	0.321	0.388	0.176	0.319	0.302	0.346	0.236	0.211	0.242	0.191	0.063	-	36.3	35.1	50.0	46.0	MD
0.507	0.310	0.351	0.431	0.218	0.338	0.344	0.405	0.257	0.274	0.356	0.254	0.164	0.140	-	3.2	15.5	13.2	VW
0.540	0.326	0.406	0.460	0.226	0.340	0.403	0.406	0.316	0.333	0.428	0.298	0.225	0.193	0.167	-	15.4	12.2	VD
0.516	0.359	0.444	0.507	0.276	0.416	0.406	0.464	0.351	0.337	0.364	0.272	0.184	0.180	0.222	0.279	-	4.7	GW
0.501	0.313	0.442	0.488	0.258	0.403	0.358	0.425	0.264	0.264	0.330	0.200	0.144	0.123	0.222	0.256	0.142	-	GD

Table S3. The F-values and their significance analysed by means of two-way ANOVA for the effects of habitat and location and their interaction on the first (FAC 1) and second (FAC 2) component scores generated from the the total biomass, root to shoot ratio, stem height, branch number, thickness of the stem, internode length, leaf size and SLA through the PCA analysis on all the nine population pairs under control conditions. The significance levels are indicated as: *** p < 0.001, ** p < 0.01, * p < 0.05, $ p< 0.1, ns p > 0.1. Significant values (p < 0.05) are in bold.

		F-values	
	d.f.	**FAC1**	**FAC2**
Habitat (T)	1	$3.678^{\$}$	0.026^{ns}
Location (L)	8	**2.176***	1.135^{ns}
H×L	8	0.882^{ns}	1.460^{ns}

Table S4. Genetic differentiation for AFLP markers (Φ_{PT}) and quantitative traits (Q_{ST}) between population pairs of *S. dulcamara* across nine locations. Q_{ST} estimates are averages over total biomass, root to shoot ratio, stem height, internode length, branch number, stem thickness, leaf size and specific leaf area.

Location	**Q_{ST}**	**Φ_{PT}**
Schiermonnikoog	0.082	0.288
Ameland	0.066	0.124
Texel	0.012	0.175
Callantsoog	0.062	0.394
Castricum	0.028	0.168
Zandvoort	0.028	0.352
Meijendel	0.104	0.063
Voorne	0.051	0.167
Goeree	0.081	0.142

Table S5. The F-values and their significance analysed by means of two-way ANOVA for the effects of habitat and treatment (flooding and drought) and their interaction on the first (FAC 1) and second (FAC 2) component scores generated from the responses of the total biomass, root to shoot ratio, stem height, branch number, thickness of the stem, internode length, leaf size and SLA of a population to flooding and drought through a PCA analysis on all the nine population pairs. The significance levels are indicated as: *** $p < 0.001$, ^{ns} $p > 0.1$. Significant values ($p < 0.05$) are in bold.

		F-values	
	d.f.	**FAC 1**	**FAC 2**
Habitat (T)	1	0.765^{ns}	1.867^{ns}
Treatment (T)	1	**2.176^{***}**	0.402^{ns}
H×T	1	0.305^{ns}	0.132^{ns}

Chapter 3

Life cycle stage and water depth affect flooding-induced adventitious root formation in the terrestrial species *Solanum dulcamara*

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Published in Annals of Botany (2015), 116:279-290

Abstract

Flooding can occur at any stage of the life cycle of a plant, but often adaptive responses of plants are only studied at a single developmental stage. We anticipated that juvenile plants may respond differently than mature plants, as the amount of stored resources may differ and morphological changes can be constrained. Moreover, different water depths may require different strategies to cope with the flooding stress, the expression of which may also depend on developmental stage. Here, we investigated whether flooding-induced adventitious root formation and plant growth were affected by flooding depth in *Solanum dulcamara* plants at different developmental stages. Juvenile plants without pre-formed adventitious root primordia and mature plants with primordia were subjected to shallow flooding or deep flooding for five weeks. Plant growth and the timing of adventitious root formation were monitored during the flooding treatments. We found that adventitious root formation in response to shallow flooding was significantly constrained in juvenile *S. dulcamara* plants compared to mature plants, and delayed by deep flooding compared to shallow flooding. Complete submergence suppressed adventitious root formation until up to two weeks after shoots restored contact with the atmosphere. Independent of developmental stage, a strong positive correlation was found between adventitious root formation and total biomass accumulation during shallow flooding. The potential to deploy an escape strategy (i.e., adventitious root formation) may change throughout a plant's life cycle, and is largely dependent on flooding depth. Adaptive responses at a given stage of the life cycle thus do not necessarily predict how the plant responds to flooding in another growth stage. As variation in adventitious root formation also correlates with finally attained biomass, this variation may form the basis for variation in resistance to shallow flooding among plants.

Introduction

Plant adaptation to flooding has been well reviewed over the last decades (Armstrong *et al.*, 1994*a*; Blom and Voesenek, 1996; Voesenek *et al.*, 2006; Perata *et al.*, 2011). Species may differ in their adaptive responses, explaining a species' tolerance to this stress, and even within a species, responses may vary among populations depending on the flooding characteristics of the habitat (Lessmann *et al.*, 1997; Lenssen *et al.*, 2004). This variation has been used for breeding purposes, resulting in rice showing a wide variation in adaptive shoot elongation among cultivars that are grown from irrigated to deepwater conditions (Kende *et al.*, 1998; Sauter, 2000).

The potential to respond to the environment may be constrained by the developmental stage of the plant (Novoplansky *et al.*, 1994; Watson *et al.*, 1995; Weinig, 2000; Groeneveld and Voesenek, 2003; Chen *et al.*, 2011). Relatively little is known of such an interaction between developmental stage and flooding responses, i.e., if the optimal strategy to survive flooding changes depending on life stage of the plant, and whether the optimal strategy differs between flooding depths.

Wetland plant species typically display various physiological and morphological adaptations when flooded (Armstrong *et al.*, 1994*a*; Blom and Voesenek, 1996). Oxygen shortage is the main problem that plants face in these conditions (Colmer, 2003), restraining aerobic respiration and causing a switch to the less efficient anaerobic respiration pathways (Jackson, 1985; Voesenek et al., 2006). Species can employ a quiescence strategy, and stop growing, thereby slowing down energy requirements, to allow carbohydrate reserves that fuel respiration to last longer (Nabben *et al.*, 1999; Almeida *et al.*, 2003; Lee *et al.*, 2009; Striker *et al.*, 2012). By contrast, "escapers" invest into shoot elongation (Akman *et al.*, 2012; Striker *et al.*, 2012), aerenchyma formation and new adventitious roots to improve internal aeration during flooding and alleviate the oxygen shortage (Colmer, 2003). Examples are shoot-elongating species like deepwater rice and *Rumex palustris* (Setter and Laureles, 1996; Jackson and Ram, 2003; Voesenek *et al.*, 2006; Jackson, 2008; Chen *et al.*, 2009). These species rapidly elongate their shoots to above the water surface during submergence, thus regaining leaf-air contact. The original root system less easily escapes from the flooding-induced oxygen deficit (Sauter, 2013), but many wetland species develop adventitious roots at the shoot base or on the stem that replace the function of the primary root system, such as water and nutrient uptake, and anchoring (Lorbiecke and Sauter, 1999). Aerenchyma connects these new roots with the shoot, and circumvents potential diffusion

barriers such as the shoot-root junction and the taproot (Sauter, 2013). Additionally, underwater photosynthesis may positively contribute to an improved oxygen and carbohydrate status of submerged plants, particularly in wetland species (Mommer & Visser, 2005; Colmer & Pedersen, 2008; Colmer *et al.*, 2011). The coordinated development of these contrasting strategies allows survival in different habitats, i.e. shallow and long-term flooding for the escapers and deep short-term flooding for the plants adopting a quiescence strategy (Voesenek *et al.*, 2004).

Although different flooding conditions may require different strategies (Manzur *et al.*, 2009), the developmental stage very likely limits which strategy a plant can actually realize. In general, plants at a more mature developmental stage have greater storage of carbon resources (Groeneveld and Voesenek, 2003) that can be exploited for fuelling fermentation or morphological changes (Huber *et al.*, 2012), than those at a young developmental stage. For instance, adult *Itea* plants survived better and had higher growth rates than juvenile plants, independent of flooding depth (Anderson *et al.*, 2009). On the other hand, young tissues may have higher plasticity than those that are more mature. For instance, the flooding-induced shoot elongation of *Rumex palustris* was largely dependent on the developmental stage of the leaf, with younger leaves having higher elongation potential (Chen *et al.*, 2009). Since selection in natural environments acts on all life stages of a plant species (McGraw and Antonovics, 1983), it is important to consider these potentially differing responses between juvenile and mature plants.

The impact of a given flooding depth depends on plant size as juvenile plants are relatively deeper submerged than taller mature plants. For terrestrial species, it is crucial to maintain a functional root system during flooding by maintaining oxygen diffusion from the emerging shoot to the submerged root (Sauter, 2013). However, deep flooding leading to complete submergence prevents direct contact to the atmosphere, thus resulting in malfunctioning of the aerenchyma (Voesenek *et al.*, 1993). Some species appear to adjust their strategy depending on flooding depth. For instance, *Lotus tenuis* switches from an escape to a quiescence strategy by ceasing shoot elongation when completely submerged (Manzur *et al.*, 2009). However, plants at very young age may not be able to respond appropriately when completely submerged as juvenile plants may simply be too small to be able to reach the water surface (Voesenek *et al.*, 1993). Furthermore, the low amount of carbohydrate reserves in juvenile plants may not be sufficient to be invested in either escaping shoot elongation or long-term quiescent metabolism. Consequently, unlike mature plants, plants at early

developmental stage may be unable to invest into either strategy and thus cannot survive prolonged complete submergence.

To study the effect of developmental stage on strategy deployment we used *Solanum dulcamara,* a perennial Eurasian species in the Solanaceae family, which is common on wet sites that are regularly flooded and forms a considerable number of adventitious roots in response to flooding (Dawood *et al.*, 2013; Visser *et al.*, 2015). Since this species occurs in a broad range of habitats along a hydrological gradient, plants originating from both wet and dry habitats were included to cover the full ecological amplitude of the species. In this study, we focused on flooding-induced adventitious root formation of *S. dulcamara* plants at two developmental stages under different flooding depths, to investigate how flooding depth and different timing of flooding in the life cycle of plants affect the performance of these plants. We hypothesized that plants in different developmental stages may respond differently to various flooding depths, because juvenile plants may be restrained in the expression of adaptive traits. This would result in a more negative impact of flooding on performance.

Materials and methods

Plant material

In 2012, seeds of *Solanum dulcamara* L. were collected at the North Sea island Texel (53° 7′ 24″ N, 4° 47′ 10″ E) and at the more southern coastal location Voorne (51° 51′ 2″ N, 4° 4′ 29″ E). For each of these locations, seeds were sampled in a wetland population at the shores of freshwater dune lakes, and in a dryland population at dry primary sand dunes well above the seasonally flooded dune slacks. The distance between these wet and dry habitats was at least 1 km. Seeds were cleaned, dried at room temperature and then stored at 4 °C.

Experimental design

The effect of developmental stage on flooding response was investigated by comparing juvenile and mature plants. At the start of the experiment, plants at the more mature developmental stage (8 weeks old mature plants, mean stem height 53.3 ± 3.1 cm, mean number of adventitious root primordia 19 ± 0.8, n = 4) had already developed a woody stem and had formed visible adventitious root primordia, whereas plants at the juvenile developmental stage (4 weeks old juvenile plants, mean stem height 5.9 ± 0.3 cm, n = 4) had not yet developed a woody stem and had no pre-formed root primordia on the

Figure 1. Pictures of the stems of 4 (A) and 9 (B) weeks old *S. dulcamara* plants. No adventitious root (AR) primordia were present on the stems of the 4 weeks old plant, whereas 6 AR primordia (the white spots indicated by the black arrows) can be discerned upon visual inspection on the stems of the 9 weeks old plant. Scale bars: (A) = 0.25 cm; (B) = 0.4 cm.

stem. These root primordia (from which adventitious roots develop) can only be visually discerned in plants that are older than six weeks (Fig. 1; pictures of stems containing either no or several primordia for 4 and 9 weeks old *S. dulcamara* plants, respectively). These primordia stay dormant on the stem until triggered by flooding (Lorbiecke & Sauter, 1999).

Both mature and juvenile developmental stages were subjected to control (drained), shallow flooding and deep flooding treatments. Plants under control conditions were given sufficient water to replenish evapotranspiration. In the shallow flooding treatment, the water level was kept at 10 cm and 3 cm above the soil for mature and juvenile plants, respectively, thus submerging at least 15 % and no more than 50 % of the plant's height. In deep flooding, water levels were maintained at 75 cm, and all juvenile plants and approximately 50 % of the mature plants were completely submerged at the start of the experiment.

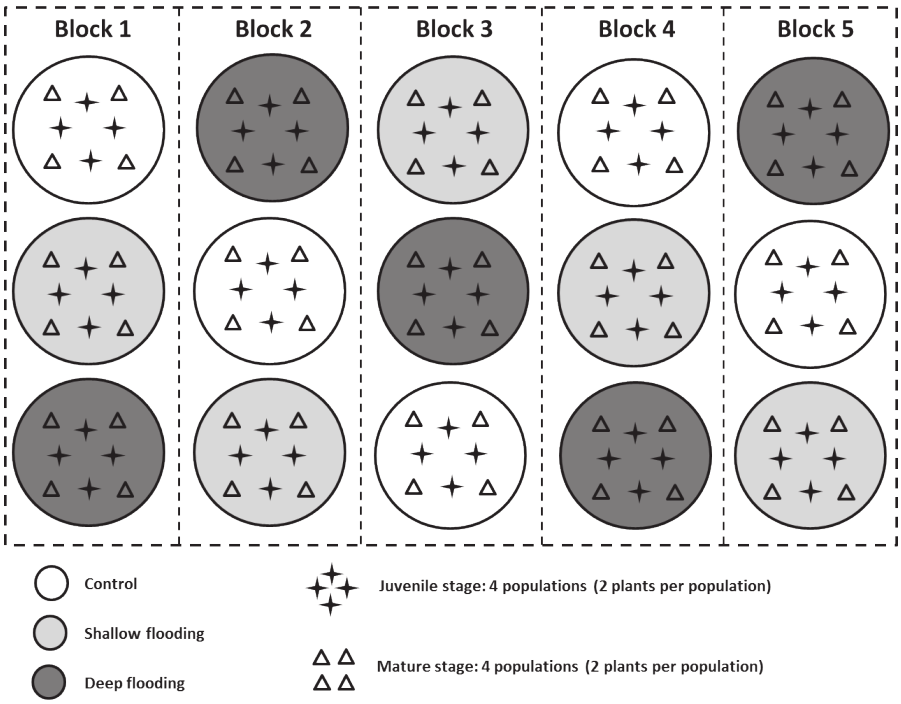

Figure 2. Schematic representation of the experimental design. Juvenile and mature *S. dulcamara* plants originating from two habitats in two locations (4 populations) were subjected to control (open circle), shallow flooding (shaded circle in light grey) and deep flooding (shaded circle in dark grey). Two plants per developmental stage per population were randomly allocated to each of the fifteen non-transparent containers, resulting in a total of 30 plants per developmental stage per population. The three containers comprising each of the three treatments in a row were treated as a block.

In the mature plants that were only partially submerged, the stems extended 0.2-23.5 cm above the water surface at the start of the treatments. These initially fully submerged plants were not prevented from reaching the water surface throughout the experiment. As initial conditions were thus different for mature plants depending on their size, this provided the opportunity to explore the effect of continued contact with the atmosphere in deep flooding on plant performance, versus having to restore this contact by elongation.

For each developmental stage, 150 seeds from each population were sown in seed trays with cells of 3.5 × 3.5 × 4 cm (length × width × depth) filled with commercial sowing compost (Horticoop substrate, Lentse potgrond & Slingerland potgrond, Cuijk, the Netherlands), and subsequently kept at 4 ˚C in dark to break dormancy. After two weeks, seed trays were transferred to the greenhouse and covered with transparent plastic foil to reduce water

loss. Plants were defined to be 0 weeks old at this time point. Two weeks after germination, within each developmental stage, 100 seedlings from each location and habitat combination were selected for homogeneity and transplanted into individual pots of 1.5 L. Nutrient-poor soil consisting of 70% sand and 30% clay supplied with 4 g L^{-1} slow release fertilizer (Osmocote® Exact Standard, NPK 15-9-12+2 MgO+tracing elements, release time 5-6 months, Everris International B.V., Geldermalsen, the Netherlands) was used as a substrate. One week after potting, 60 ml nutrient solution (2 g L^{-1} Kristalon in tapwater, Yara International ASA, Vlaardingen, the Netherlands) was added to each pot to avoid nutrient limitation at the onset of the experiment.

For each developmental stage, ten plants per population were randomly assigned to each treatment and in a block design placed in black containers (height 80 cm, diameter 100 cm). To reduce edge effects of the container, plants were placed 15 cm away from the wall of the container. Fifteen containers were allocated to the three treatments and three containers in a row covering each treatment were subsequently treated as a block (Fig. 2). The experiment was conducted outside in an open non-shaded area for five weeks from 7 May to 10 June 2013. The average daily maximum and minimum air temperature were 18.8 ± 0.9 and 8.8 ± 0.5 ˚C, respectively, and the average daily maximum and minimum relative humidity were 97.6 ± 0.9 and 59.3 ± 2.9 %, respectively. The water temperature was 2 and 4 ˚C higher in shallow flooding and deep flooding than the air temperature, respectively. Throughout the experiment, water was added to or removed from the containers depending on precipitation and/or evaporation, to keep the plants under control condition well-watered but drained, and to keep the water level constant in the shallow flooding and deep flooding treatments. Juvenile and mature plants were placed together in the same containers. For the shallow flooding treatments, the pots containing juvenile plants were raised by 7.0 cm, so that the water level was 3 cm above soil level. The stagnant floodwater remained clear and free of algae during the entire experiment.

To investigate whether juvenile plants can deploy a quiescence strategy in complete submergence, an additional greenhouse experiment was performed. In this experiment, the survival over time of *S. dulcamara* seedlings completely submerged in either light or dark conditions was monitored. The seeds of a wetland population originating from Voorne, were placed on a moist filter paper for germination. After germination seedlings were transferred to 0.5 L pots filled with nutrient-poor soil consisting of 70% sand and 30% clay. Three weeks old seedlings (initial stem height 4.3 ± 0.3 cm, n=7) were completely submerged in black containers (50 cm high, volume 90 L) filled with tap water.

Half of the containers were fully wrapped with black, light impermeable, plastic sheets and the other half were fully wrapped with transparent plastic sheets. The plastic sheets were positioned 20 cm above the water surface to allow for gas diffusion. For each treatment (light and dark conditions), the survival of 14 plants was scored after 1, 2 and 4 weeks of flooding, respectively.

Measurements

Before the onset of the flooding treatments, stem length and leaf number of both, the main stem and side branches, the node number and the number of root primordia of the main stem were quantified for each plant. For each developmental stage, ten extra plants per population were harvested to determine the initial biomass.

Throughout the experiment, the date on which the shoot extended above the water surface and the date when the first adventitious root emerged were recorded for each plant. Adventitious roots were considered to be emerged if the root primordium elongated for > 2 mm. At harvest, the adventitious roots were separated from the stem and counted. The original roots were carefully washed free of soil with tap water. The length of the main stem and the side branches were measured, and the leaf number and node number on the main stem and side branches were counted. Leaf decay and abscission occurred in both flooding treatments, especially in the deeply flooded plants, and only viable turgescent leaves were included in the measurements. The different plant parts were weighed after drying at 70°C for 72 hours.

Data analysis

Total biomass, stem height and adventitious root number and biomass were log10 transformed to reduce heteroscedasticity. Data were analyzed using a mixed model nested analysis of variance (ANOVA), with developmental stage, treatment and location as main effects, and habitat of origin nested within location. To investigate the effects of treatment, location and habitat in each developmental stage, we performed the nested ANOVA for each developmental stage separately followed by a Tukey Post Hoc test, with treatment and location as main effects, and habitat of origin nested within location. Since juvenile plants did not form any adventitious root in deep flooding, the analysis on adventitious root number and biomass was only performed on plants subjected to shallow flooding. As most adventitious roots of mature plants were produced from the pre-formed root primordia in the experiment, the number of root primordia was added as a covariate to examine the effects of initial root primordia as well as treatment, location and

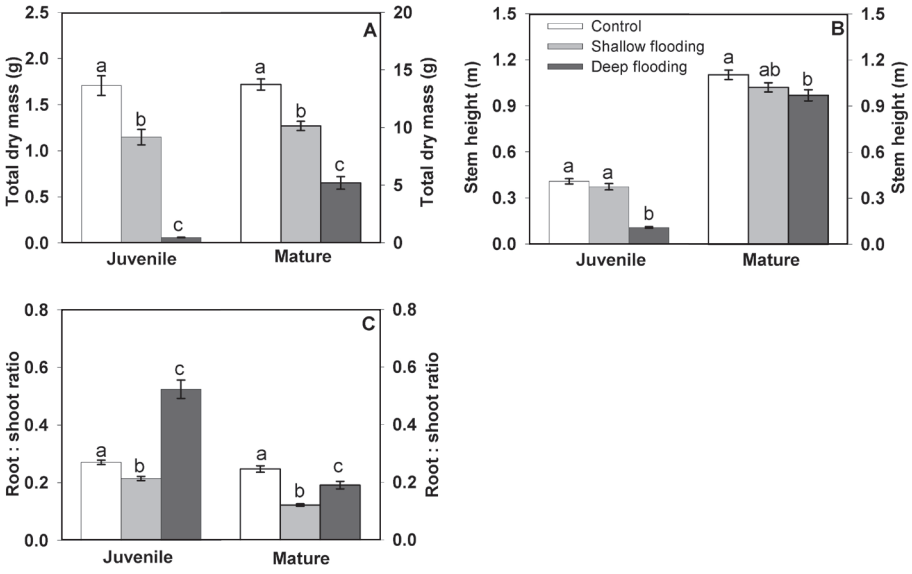

Figure 3. Total biomass (A), stem height (B) and root to shoot ratio (C) of *S. dulcamara* plants at juvenile and mature developmental stages in control, shallow flooding and deep flooding treatments. The initial stem height and total biomass were 5.93 ± 0.32 and 53.32 ± 0.31 cm, 0.12 ± 0.01 and 2.91 ± 0.15 g for juvenile and mature plants, respectively. The left y-axis refers to juvenile plants and the right y-axis to mature plants. Means ± s.e. (n = 4 populations for each treatment) that do not share a letter are significantly different by a Tukey's post-hoc test ($p \leq 0.05$) within each developmental stage.

habitat nested within location on the adventitious root formation in mature plants. Block was also included in ANOVA as a random factor. Separate regression analyses were performed to investigate the relationship between adventitious root biomass and initial stem height, adventitious root biomass and total biomass, and adventitious root number and total biomass for each developmental stage in shallow flooding. Regression analysis was also used to investigate the relationship between the time until shoots reached the water surface and the time to form adventitious roots for mature plants in deep flooding. In the experiment investigating whether juvenile plants deploy a quiescence strategy, survival was analyzed using Chi-Square test, with duration of submergence and light availability as main effects. We also used pairwise Chi-Square tests to compare the effect of flooding duration for plants within light condition treatments. Significance levels were adjusted to 0.0170 and 0.003 instead of 0.05 and 0.01 in order to take account of performing multiple

Table 1 The results of nested ANOVAs for the effects of stage, treatment, location and habitat (location) on total biomass, stem height and root to shoot ratio of *S. dulcamara*. Degrees of freedom (d.f.) and F values and their significance are presented.

		F-values		
	d.f.	**Total dry mass**	**Stem height**	**Root/shoot ratio**
Stage	1	**2642.334*****	**5.414***	**152.045*****
Treatment	2	**610.385*****	**231.959*****	**79.731*****
Stage × treatment	2	**149.124*****	**210.992*****	**58.418*****
Location	1	0.264^{ns}	1.712^{ns}	0.575^{ns}
Stage × location	1	0.569^{ns}	**10.168****	**2.768***
Treatment × location	2	**3.929****	**3.403***	**4.08****
Stage × treatment × location	2	**3.954****	**5.574****	**4.182****
Habitat (location)	2	**2.619***	**3.531***	1.756^{ns}
Stage × habitat (location)	2	**3.037***	0.236^{ns}	0.148^{ns}
Treatment × habitat (location)	4	**2.511****	0.409^{ns}	1.096^{ns}
Stage × treatment × habitat (location)	4	1.001^{ns}	0.527^{ns}	0.432^{ns}
Block	4	1.901^{ns}	0.750^{ns}	1.375^{ns}

**** $p < 0.001$, **$0.001 < p < 0.01$, *$0.01 < p < 0.05$, $^{ns}p > 0.05$. Significant differences at $p < 0.05$ are in bold.*

comparisons. All the analyses were performed in SPSS version 20 (IBM New York, NY, USA).

Results

Plant performance and biomass allocation were significantly affected by flooding depth

Shallow flooding and deep flooding significantly decreased the total biomass of *S. dulcamara* in both developmental stages (Fig. 3A, Table 1), compared to control plants. Deep flooding led to substantially lower biomass increment than shallow flooding throughout the experiment. Compared to control plants, juvenile and mature plants subjected to deep flooding had approximately 90% and 60% lower biomass increment, respectively (Fig. 3A). Final stem height was also negatively affected by both flooding treatments (Fig. 3B). For the

Table 2 The results of nested ANOVAs for treatment, location and habitat (location) on the total biomass, stem height and root to shoot ratio of *S. dulcamara* in mature and juvenile stages. Degrees of freedom (d.f.) and F values and their significance are presented.

		F-values		
	d.f.	**Total dry mass**	**Stem height**	**Root/shoot ratio**
Mature stage				
Treatment	2	**81.692*****	**6.610*****	**37.782*****
Location	1	0.030^{ns}	**4.232***	0.894^{ns}
Treatment × location	2	0.044^{ns}	1.664^{ns}	0.395^{ns}
Habitat (location)	2	**4.849****	**17.085*****	1.019^{ns}
Treatment × habitat (location)	4	**3.267****	0.219^{ns}	2.006^{ns}
Block	4	**2.657***	**2.694***	0.419^{ns}
Juvenile stage				
Treatment	2	**664.365*****	**247.447*****	**78.861*****
Location	1	0.786^{ns}	0^{ns}	1.914^{ns}
Treatment × location	2	**7.660*****	**8.531*****	**5.275****
Habitat (location)	2	0.915^{ns}	2.182^{ns}	0.938^{ns}
Treatment × habitat (location)	4	0.325^{ns}	0.240^{ns}	0.396^{ns}
Block	4	0.390^{ns}	0.236^{ns}	1.581^{ns}

*** $p < 0.001$, **$0.001 < p < 0.01$, *$0.01 < p < 0.05$, ^{ns} $p > 0.05$. Significant differences at $p < 0.05$ are in bold.

mature developmental stage, shallow flooding and deep flooding both resulted in shorter plants, with no clear differences between these flooding depths (Fig. 3B). For the juvenile developmental stage, control plants were only slightly taller than shallowly flooded plants, but almost four times as tall as the deeply flooded plants (although these latter plants still had an average increment of stem height of 5.8 cm) (Fig. 3B, Table 2).

Flooding also affected biomass allocation patterns. Root to shoot ratio (adventitious roots were excluded) was reduced by flooding except for the completely submerged juvenile plants (Fig. 3C). Generally, mature flooded plants had a significantly lower root to shoot ratio than those under drained control conditions (Fig. 3C, Table 2). However, in mature plants, shoot growth was less limited by shallow flooding than by deep flooding, resulting in a smaller reduction of the root to shoot ratio in plants subjected to deep flooding than in those subjected to shallow flooding (Fig. 3C). Surprisingly, the root to shoot ratio of juvenile plants was substantially increased by deep flooding

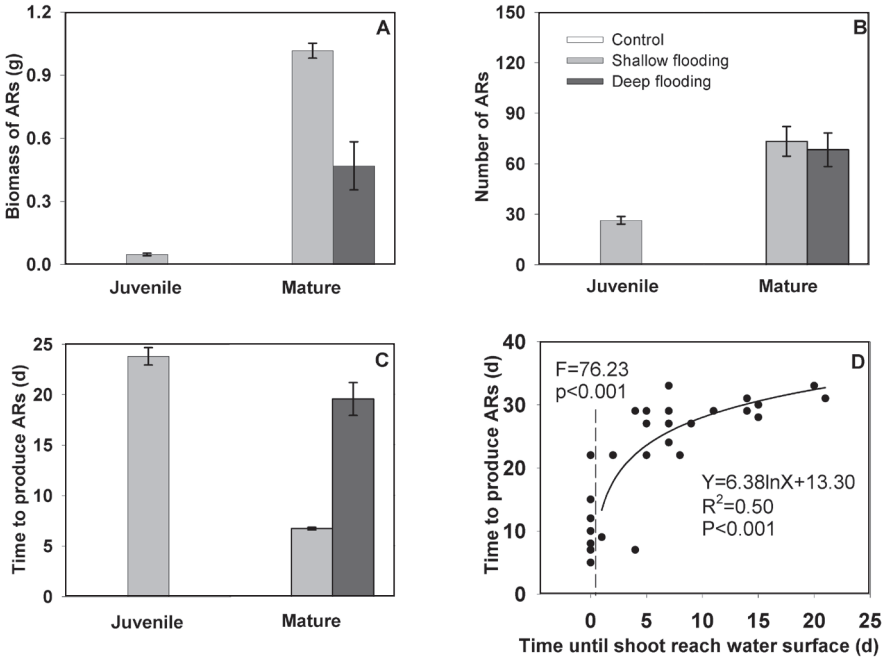

Figure 4. Adventitious root (AR) biomass (A), number of adventitious roots (B), time to produce adventitious roots (C) of juvenile and mature *S. dulcamara* plants in control, shallow flooding and deep flooding treatments, and the relationship between the time to produce adventitious roots and the time until shoot reach the water surface (D) of mature plants in deep flooding.

(Fig. 3C), which was probably due to the reduced shoot biomass, resulting from leaf abscission under water, rather than an increase in root biomass.

Adventitious root formation was affected by flooding depth and developmental stage

Mature plants formed adventitious roots under both flooding conditions, whereas juvenile plants only produced adventitious roots if subjected to shallow flooding (Fig. 4A, B). None of the juvenile plants subjected to deep flooding formed adventitious roots throughout the experiment. The timing of the onset of adventitious root formation differed significantly between juvenile and mature plants subjected to shallow flooding; under these conditions mature plants started to form adventitious roots about 20 days earlier (Fig. 4C). For completely submerged mature plants in deep flooding, the onset of adventitious root formation also depended on the time when the shoot extended above water surface. Plants that were extending above the water

Table 3 The results of nested ANOVA for the effect of treatment, location, habitat (location) and initial root primordia on the biomass of adventitious roots (AR dry mass) and number of adventitious root (AR number) of *S. dulcamara* from mature developmental stage. Degrees of freedom (d.f.) and F values and their significance are presented.

		F-values	
	d.f.	**AR dry mass**	**AR number**
Treatments	1	**25.124*****	0.181^{ns}
Location	2	0.659^{ns}	0.037^{ns}
Treatment × location	1	2.416^{ns}	0.78^{ns}
Habitat (location)	1	0.117^{ns}	1.283^{ns}
Treatment × habitat (location)	2	1.502^{ns}	1.166^{ns}
Root primordia	1	**10.342****	**16.909*****
Location ×root primordia	1	1.438^{ns}	0.399^{ns}
Root primordia × habitat (location)	2	0.317^{ns}	0.184^{ns}
Block	4	1.307^{ns}	0.546^{ns}

*** $p < 0.001$, **$0.001 < p < 0.01$, *$0.01 < p < 0.05$, ^{ns} $p > 0.05$. Significant differences at $p < 0.05$ are in bold.

surface at the start of deep flooding formed adventitious roots on average nine days after the onset of flooding (Fig. 4D). However, plants that were entirely under water at the start of flooding produced the first adventitious roots up to 30 days after the onset of flooding, which was 15-20 days after they reached the water surface (Fig. 4D).

The number of pre-formed root primordia significantly affected adventitious root number and biomass (Table 3). Mature plants in deep flooding had much lower adventitious root biomass and almost the same number of adventitious roots compared to plants in shallow flooding (Fig. 4A, B, Table 3), indicating that not root initiation but root development was negatively affected by deep flooding.

Plant biomass after shallow flooding correlated with adventitious root formation

Stem height at the onset of the experiment had only a very small effect on adventitious root formation (Fig. 5A, B), suggesting that within a given developmental stage, adventitious root formation did not depend on initial size of the plant at the start of treatments. However, the final biomass of the

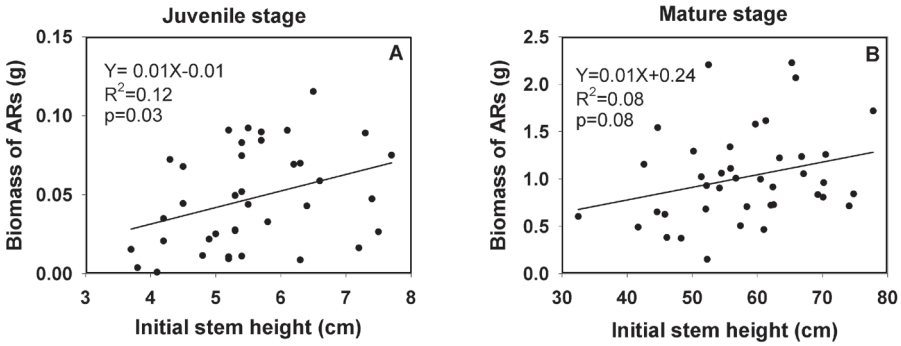

Figure 5. The relationship between initial stem height and biomass of adventitious roots (ARs) in juvenile (A) and mature *S. dulcamara* plants (B) subjected to shallow flooding.

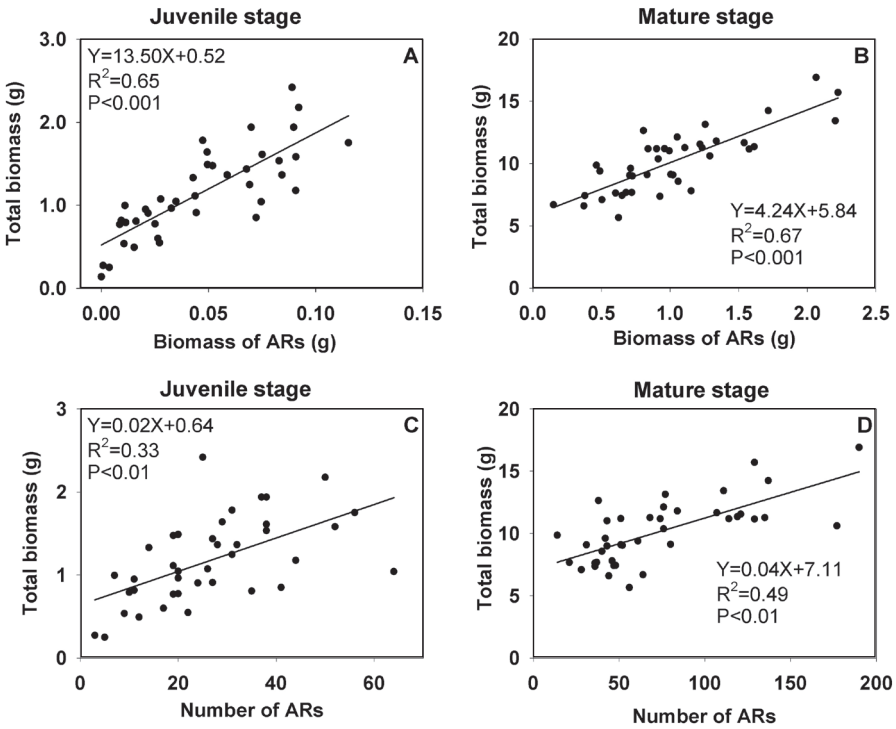

Figure 6. The relationship between the biomass of adventitious roots (ARs) and total biomass of juvenile (A) and mature *S. dulcamara* plants (B), and the relationship between the number of adventitious roots and total biomass of juvenile (C) and mature plants (D) subjected to shallow flooding.

adventitious roots was positively correlated with the biomass of the entire plant after treatment in both developmental stages (Fig. 6A, B). Similarly, the number of adventitious roots was positively correlated with total biomass of the plants (Fig. 6C, D), which might reflect a positive effect of adventitious roots on plant performance during flooding.

Habitat and location of populations had only limited effects on flooding responses

In mature plants, a significant interaction was found between the effects of treatment and habitat nested within location on the total biomass (Tables 1, 2), as plants from the dry habitat accumulated 55% more total biomass than those from the wet habitat in Voorne in deep flooding treatment (Supplementary Data Fig. S1). In juvenile plants, treatment and location had significantly interacted in determining total biomass, stem height and root to shoot ratio (Tables 1, 2). Plants originating from Voorne had approximately 40% less total biomass and 22% shorter stem height in shallow flooding treatment, but 30% more biomass and 26% taller stem height in deep flooding treatment than those originated from Texel (Supplementary Data Fig. S2). The plants originated from Voorne also had approximately 30% smaller root to shoot ratio than those originated from Texel in deep flooding treatment (Fig. S2).

Light availability was important for plant survival in complete submergence

To investigate if juvenile plants possessed an alternative strategy for surviving complete submergence, i.e., a quiescence strategy, we tested if these plants could withstand submergence in complete darkness. Plants completely submerged in the light had 100% survival after four weeks of flooding, whereas the survival rate of plants completely submerged in the dark decreased to approximately 20% within two weeks of flooding (Fig. 7). After four weeks of flooding, all plants subjected to complete submergence in the dark died ($p<0.01$, Fig. 7). This suggested that underwater photosynthesis was needed to supply the plants with carbohydrates and/or oxygen, and no true quiescence strategy was present.

Discussion

The risk of flooding events will increase in the near future due to global warming (IPCC, 2007). Economic losses may therefore become substantially higher as flooding causes high mortality and considerable damage to many crop

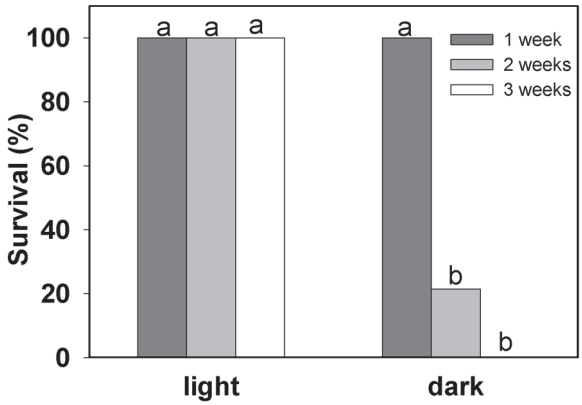

Figure 7. The survival percentage of juvenile plants completely submerged in light and in dark at the end of the first, second and fourth week. Different letters indicate significant differences at p = 0.017, n = 14 (Significance levels were adjusted to 0.0170 and 0.003 instead of 0.05 and 0.01 to take account of performing multiple comparisons).

species (Normile, 2008; Bailey-Serres *et al.*, 2012). However, wild plant species occurring in flood-prone habitats survive flooding much better than crop species, due to the ability to show morphological and anatomical acclimations, e.g., shoot elongation, adventitious rooting and aerenchyma formation (Visser *et al.*, 1996; Chen *et al.*, 2009). Studying these adaptive responses of natural wetland species may provide knowledge that can help to introduce flooding tolerance into crops. Here, we show that adventitious root development induced by flooding is constrained at the juvenile stage of *Solanum dulcamara* plants, and that deep flooding delays adventitious root formation compared to shallower flooding. These results indicate that it is important to study impact of environmental stress and plant's adaptive responses at different stages of the life cycle, as responses at a given developmental stage not necessarily predict how the plant responds in another growth stage.

Developmental stage affects the timing of adventitious root emergence

The timing of emergence of adventitious roots varies among species but is generally quick, from within 12 h in deepwater rice (Lorbiecke and Sauter, 1999) to 1-2 days in sunflower (Wample and Reid, 1975; Wample and Reid, 1978), tomato (Vidoz *et al.*, 2010) and *Rumex* species (Visser *et al.*, 1996). These adventitious roots develop from new primordia that are rapidly formed

in response to flooding (Vidoz *et al.*, 2010), or from pre-formed primordia which are present on the stem and remain dormant until triggered by flooding (Lorbiecke and Sauter, 1999). The latter is the case in mature *S. dulcamara* plants, which typically show numerous root primordia on the woody base of the stem (Dawood *et al.*, 2013). In juvenile plants of this species, however, root primordia still need to be developed upon flooding, potentially explaining the strong delay in adventitious root formation observed in our experiment. Mechanisms of outgrowth of adventitious roots (referred to as nodal or crown roots in *Poaceae* species) are well-studied in rice (Steffens and Sauter, 2005; Steffens *et al.*, 2006; Steffens and Sauter, 2009). In this species, genes involved in adventitious root formation were earlier expressed upon flooding when root primordia were on older stem nodes, leading to faster root outgrowth (Lorbiecke and Sauter, 1999). A similar mechanism may be an additional reason for the difference in timing of adventitious root development between juvenile and mature plants in *S. dulcamara.* Furthermore, the outgrowth of adventitious roots in juvenile plants may have been more constrained by carbohydrate limitation than in mature plants. A positive relationship between carbohydrate content and adventitious root development was shown earlier by Druege *et al.* (2004) for cuttings of *Pelargonium*, and improved adventitious rooting capacity by carbohydrate supply was found in cuttings of *Eucalyptus saligna* Smith and *Eucalyptus globulus* Labill (Corrêa *et al.*, 2005), suggesting that accumulation of carbohydrate reserves at later life stages may have accelerated adventitious root formation in our experiment.

In shallowly flooded *S. dulcamara*, adventitious root formation started seven days after the onset of flooding in the current study, which is five days later than in previous experiments on this species (Dawood *et al.*, 2013). This relative delay in the formation of adventitious roots might seem surprising, as Dawood *et al.* (2013) also used mature plants in their study. However, floodwater temperature was substantially lower in the current experiment (12.9 vs 20 °C), which may have slowed down metabolism and growth rate (van Eck *et al.*, 2005). Moreover, higher oxygen solubility in cold water (van Eck *et al.*, 2005) may also have delayed the signal of oxygen deficit in the current experiment. Genotypic differences in rate of outgrowth can also not be excluded, although observations on plants from a larger set of populations did not reveal substantial differences in timing of development of adventitious roots (personal observation, Q. Zhang). These results indicate that different mechanisms such as developmental stage and water temperature can contribute to variation in adventitious root formation. Whether such variation has ecological consequences for a species when confronted with environmental stress needs further attention.

Complete submergence suppresses adventitious root formation

Complete submergence, resulting in stronger oxygen deficit than partial submergence (Armstrong *et al.*, 1994*b*; Rijnders *et al.*, 2000; Mommer and Visser, 2005), may cause severe stress even in flood-tolerant terrestrial plants. This stress may be largely alleviated in clear water, where light levels remain sufficiently high to enable underwater photosynthesis (Mommer *et al.*, 2006; Pedersen *et al.*, 2013). Still, the formation of adventitious roots in our experiment was suppressed in *S. dulcamara* plants when fully submerged in clear water. This inhibition was relieved after shoots extended above the water surface in the course of the experiment, which is a similar response as shown by fully submerged *Rumex maritimus* and *R. palustris* plants (van der Sman *et al.*, 1993). The most obvious reason for this inhibition of root outgrowth is the assumed low aerobic respiratory rate and thus low ATP production in completely submerged plants due to limited diffusion of oxygen into the plant, causing processes demanding high energy availability, such as cell division, to cease. Also, the conversion of starch into sugars that fuel respiration may be hampered at low oxygen concentrations (Perata *et al.*, 1997), which in turn inhibits growth. Some species, however, such as *Meionectes brownii* and *Cotula coronopifolia*, can produce adventitious roots both under partial flooding and during complete submergence (Rich *et al.*, 2012). In those species, maintenance of underwater photosynthesis may have contributed to a high endogenous oxygen concentration during day time, facilitating energy generation through aerobic respiration. *S. dulcamara* plants, however, lost most submerged leaves in our experiment, thus constraining their capacity of underwater photosynthesis. One may expect then that after the shoot restores contact with the atmosphere, the renewed oxygen supply to the adventitious root primordia would result in immediate outgrowth. This was not the case, as adventitious roots still took up to 2-3 weeks to emerge. This may indicate that the production of adventitious roots is under regulatory control and actively inhibited, rather than just constrained by oxygen shortage.

Juvenile plants failed to employ an alternative strategy in complete submergence

Juvenile plants survived complete submergence without developing adventitious roots, indicating that these plants may deploy an alternative strategy, i.e., quiescence (Bailey-Serres and Voesenek, 2008). This would require juvenile plants to also be able to survive complete submergence without photosynthesis, i.e., in turbid water or in the dark. However, juvenile plants showed as much as 80% mortality after two weeks of complete submergence

in the dark, as opposed to no mortality at all in the light. Apparently, these juvenile plants did not have sufficient carbohydrate stores, or could not access these reserves, to survive two weeks of flooding-induced anaerobiosis, and largely depended on underwater photosynthesis to maintain basic physiological functions. This indicates that lack of adventitious root formation in these young plants is not a sign of the proposed quiescence strategy.

Adventitious roots improve plant growth during shallow flooding

Developing abundant adventitious roots may result in superior plant growth during flooding, as severing adventitious roots largely reduced plant growth under these conditions (Tsukahara and Kozlowski, 1985; Javier, 1987; Rich *et al.*, 2012). In our experiment, juvenile and mature *S. dulcamara* plants subjected to shallow flooding showed a positive correlation between adventitious roots (number and biomass) and total plant biomass. This could be explained by two opposing hypotheses. First, large plants may have accumulated more resources and thereby were able to produce more adventitious roots. Alternatively, plants may have profited from forming more adventitious roots and were thus able to accumulate a higher biomass. The first hypothesis is partly confirmed, since initial stem height, as an indicator for plant biomass, did affect the adventitious root biomass after the flooding period, but this effect was relatively small, explaining merely 10% of the variation and being only significant for mature plants. The biomass produced during the flooding period, however, correlated much stronger to the number and biomass of roots formed (explaining > 65% of the variation), suggesting that plant growth during flooding directly benefited from the development of large numbers of adventitious roots. This was also shown in experiments where the number of adventitious roots was manipulated by root removal. In sunflower, the initially decreased shoot growth rate in response to flooding partly recovered in plants with normal adventitious root development, and remained low in plants whose adventitious roots were removed. Severing adventitious roots also negatively affected plant growth in two legume species (Javier, 1987) and in two herbaceous wetland species (Rich *et al.*, 2012). Comparisons of closely related species differing in adventitious root formation showed a correlation between the number of adventitious roots and the position of the species in a natural flooding gradient (for *Rumex*; Visser *et al.*, 1996), or the maintenance of high plant growth rates (in woody *Hakea* species; Poot and Lambers (2003)). In our experiment, we showed that even within a single species, natural variation in adventitious root formation may be associated with plant performance under soil flooding.

Conclusions

Our results show that juvenile plants delayed the flooding-induced plastic response of adventitious root formation more than mature plants, most likely due to the absence of already pre-formed adventitious root primordia. Root production may have further been delayed by a lower amount of stored carbohydrates, suggesting that the potential to express essential responses necessary to withstand flooding, changes throughout the life cycle of a plant. A specific adaptive response expressed at any given developmental stage does thus not necessarily predict to what extent a plant is adapted to flooding throughout its life-time and the nature of responses at other developmental stages. The fact that juvenile plants responded more slowly and suffered strongly reduced performance under deep flooding indicates that even *S.dulcamara* plants growing in permanently flooded lake shores may require drained conditions during germination and juvenile development. Moreover, flooding depth and the capacity and timing of restored shoot contact with the atmosphere greatly influenced the formation of adventitious roots, indicating that delayed contact with the atmosphere further delays root outgrowth, even after the contact with the atmosphere is regained. Furthermore, our data show that the natural variation in adventitious root formation within a species is likely to contribute to the flooding resistance of the plant. Further experiments would need to focus on the nature of this relationship.

Acknowledgements

We are grateful to Tijn Raaijmakers for performing the survival experiment. We also appreciate the assistance of technicians and students at the department, and Gerard van der Weerden and co-workers at the Nijmegen Experimental Garden. This experiment is part of the B'sweet collaborative research initiative of the Departments of Experimental Plant Ecology and Molecular Plant Physiology of the Radboud University Nijmegen. Qian Zhang was supported by China Scholarship Council grant number 201206510011.

Appendix

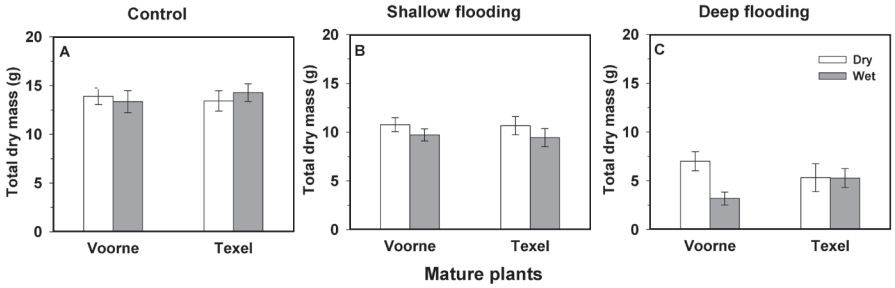

Figure S1. Total biomass of mature *S. dulcamara* plants originating from wet and dry habitats in Voorne and Texel in control, shallow flooding and deep flooding treatments (n = 10). In each treatment, the habitat effect within location was examined with a student's t test. Significant differences are indicated by star symbols (**0.001 < p < 0.01).

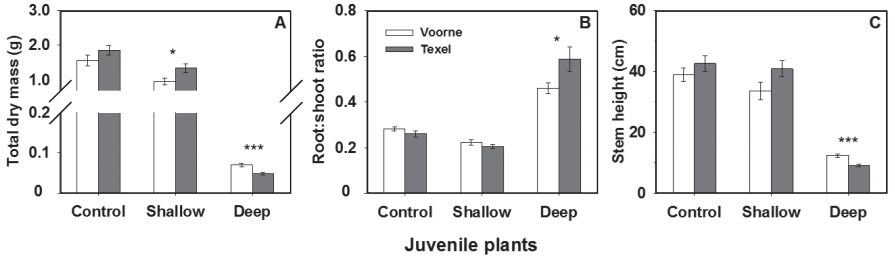

Figure S2. The total biomass, root to shoot ratio and stem height of juvenile *S. dulcamara* plants originating from Voorne and Texel in control, shallow flooding (Shallow) and deep flooding (Deep) treatments (n = 20). For each treatment, the location effect was examined with a student's t test. The significant differences are indicated by star symbols (*** p < 0.001, *0.01 < p < 0.05).

Chapter 4

Benefits of flooding-induced adventitious roots depend on the duration of submergence: linking plant performance to root functioning

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Manuscript in preparation for Annals of Botany

Abstract

Temporal flooding is a common environmental stress for terrestrial plants. Upon flooding, the belowground root system encounters severe oxygen deficiency and gradually loses its function. Adventitious roots produced by flooding tolerant plant species are generally assumed to take over the function of the sediment roots, thus potentially conferring fitness advantages in terms of enhanced plant performance during flooding. However, such contribution of adventitious roots to plant performance has hardly been quantified. Apart from the fitness benefits in terms of increased water and nutrient uptake, plants may also incur costs from the investment into adventitious root development in terms of carbohydrates, which may offset the beneficial effects of adventitious roots or even negatively influence plant performance. We tested the hypothesis that the balance between potential costs and benefits depends on the duration of flooding, as the benefits are expected to overweigh the costs in long-term, but not in short-term flooding. By manipulating adventitious root growth and measuring nutrient uptake by adventitious roots, transpiration and photosynthesis, we confirmed that adventitious roots are beneficial due to their contribution to resource uptake. These beneficial effects were only present in long-term but not in short-term flooding. This relationship can be explained by the correlation between nutrient uptake and the size of adventitious root system, which in turn depends on flooding duration. This indicates that adventitious root formation is likely to be selected for in long-term flooding. Our results revealed limited costs of adventitious root formation, suggesting that adventitious root formation is generally cheap, which makes it likely for plants to maintain this plasticity even in environments where plants are not exposed to flooding.

Introduction

Temporal flooding is a common environmental stress, even for terrestrial plants (Voesenek *et al.* 2006; Colmer & Voesenek 2009). Upon flooding, O_2 is rapidly depleted in the rhizosphere due to respiration of roots and aerobic micro-organisms, which subsequently causes loss of function of the root system as the oxygen requiring metabolic processes are strongly hampered (Sauter 2013). Many wetland plant species, however, produce adventitious roots during flooding events. These roots usually form on the stem of the plant (Jackson & Drew 1984; Rich, Ludwig & Colmer 2008; Steffens *et al.* 2012; Steffens & Rasmussen 2016), and contain aerenchyma, a highly porous tissue type that facilitates O_2 transport from the shoot into the root. This improves the internal aeration of the plant, particularly when parts of the shoot are still extending above the floodwater surface, and allows energy-dependent root functions such as water and nutrient uptake to continue (Colmer 2003). Therefore, adventitious root formation is considered an important adaptation of plants to flooding. Remarkably, empirical studies quantifying these assumed benefits of adventitious roots for plant performance are still scarce. This study aims to provide evidence for a positive contribution of adventitious roots to plant performance, and investigates if this relationship depends on flooding duration.

Previous studies particularly linked adventitious root formation to plant performance by quantifying variation in adventitious rooting among closely related species growing along a flooding gradient. These studies revealed that species from habitats characterised by different hydrological regimes differed significantly in the morphology and quantity of adventitious roots (Laan *et al.* 1989; Blom *et al.* 1994; Visser, Blom & Voesenek 1996). The flooding tolerance of species seems to be closely linked to their capability of adventitious root formation. E.g., Whiteman *et al.* (1984) investigated the flooding tolerance of 17 tropical pasture legumes, and found that species with a capacity to produce medium to large adventitious root systems to have superior flooding tolerance. The number and porosity of adventitious roots also positively correlated with the flooding tolerance of woody *Hakea* species from contrasting hydrological environments (Poot & Lambers 2003). Even a single species can be subjected to distinctively different flooding regimes if it occurs along a wider range of a flooding gradient (Lenssen *et al.* 2004; Chen *et al.* 2009). Covering such a wide range of a flooding gradient potentially selects for within-species difference in adaptive traits related to flooding tolerance, such as adventitious root development, and is thus expected to lead to genetic differentiation

along the flooding gradient in terms of flooding resistance. A previous study testing this hypothesis revealed considerable among-population variation in adventitious root formation of a plant species occurring in contrasting hydrological environments, however, surprisingly, this variation could not be linked to the local habitat types (Chapter 2). In that study, the terrestrial perennial species *Solanum dulcamara* L. (Bittersweet) which produces large numbers of adventitious roots, was used (Zhang *et al.* 2015; Chapter 2), of which eighteen populations originating from dry and wet habitats across nine different locations were subjected to partial flooding (see Chapter 2). Although neither plant performance in terms of biomass production nor adventitious root formation differed between the two types of habitat (Chapter 2), a re-analysis of the data across these 18 populations revealed a positive correlation between adventitious root biomass and the performance of the flooded plants (Fig. 1). These results indicate that while hydrological regimes under natural conditions apparently did not select for differences in adventitious root formation between habitats, overall natural variation in adventitious root formation might result in fitness differences in terms of biomass production in *S. dulcamara*. To unambiguously show the fitness benefits of adventitious roots, experiments manipulating the formation of adventitious roots coupled to measurements of fitness parameters such as plant biomass production and the associated water and nutrient uptake are needed.

Under flooded conditions, adventitious roots have been shown to play an important role in nutrient uptake (Khan, Ventura & Vergara 1982; Sauter 2013). As uptake of several mineral nutrients strongly depends on the total root surface area (Comas *et al.* 2013), the beneficial effects of adventitious roots may be more apparent in long-term flooding events where the adventitious roots have grown substantially larger than in short-term flooding. Furthermore, adventitious roots in wetland species typically grow in the floodwater column rather than in the sediment, and most of these roots may die after flooding subsides (Rich, Ludwig & Colmer 2012; Zhang *et al.* 2015). The loss of adventitious roots after short-term flooding may then negatively affect plant performance, as the initial investment into cell division and elongation during adventitious root development might have not been returned in terms of water and nutrient uptake (Takahashi *et al.* 2003). Consequently, the net benefits of adventitious roots are expected to depend on flooding duration.

In the present study, we used the species *S. dulcamara* to 1) quantify the benefits of adventitious roots during prolonged partial flooding by experimentally manipulating the number of adventitious roots formed, 2) show the contribution of these adventitious roots in nutrient and water uptake

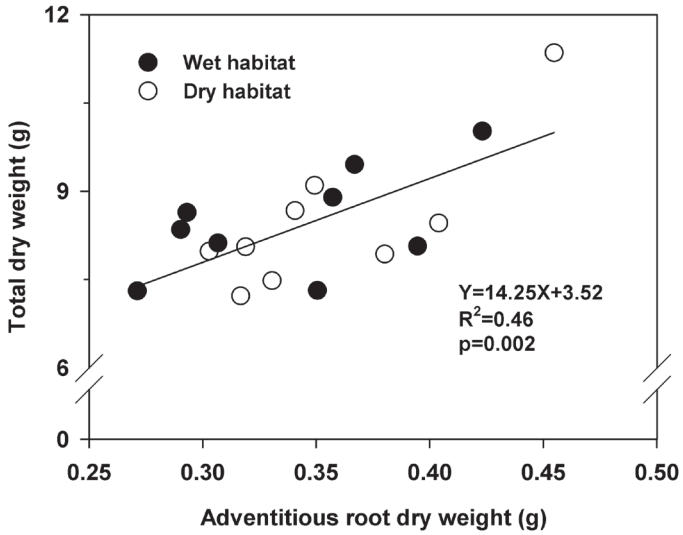

Figure 1. Relation between the dry weight of adventitious roots and the total dry weight (adventitious root biomass excluded) of the entire plant after three weeks of experimental partial flooding of plants originating from dry (open symbols) and wet (shaded symbols) habitats along the Dutch coast (original data from Chapter 2). To test the relationship between adventitious root biomass with total dry weight excluding adventitious root biomass across 18 populations of *S. dulcamara* (Chapter 2), a linear regression model was fitted with adventitious root biomass and the total dry weight as independent and dependent variables, respectively.

under these conditions, and 3) elucidate the effect of flooding duration on adventitious roots and plant performance.

Materials and methods

Species and plant material

Solanum dulcamara L. (Bittersweet) is a perennial, often climbing species (D'Agostino *et al.* 2013), native to Europe and occurring in contrasting ecological habitats, such as permanently flooded wetlands and dry sand dunes (Visser *et al.* 2015; Zhang *et al.* 2015). For a larger research programme, seeds were collected from *S. dulcamara* populations originating from either wet or dry habitats at nine locations along the coastline of the Netherlands (Chapter 2) in autumn 2012 and 2013. Detailed information about these contrasting habitats has been described in Chapter 2. The seeds were cleaned and dried at room temperature, and then stored at 4 ˚C. For the present experiments, seeds collected along a dune lake on the island of Texel were used. This population

was chosen due to its representative position in terms of adventitious root production among the 18 populations.

Prior to sowing, seeds were surface sterilized with 15 % (w:v) tri-sodium phosphate solution in water for 20 min and rinsed with abundant tap water. After sterilization, seeds were either directly sown in seed trays with cells of 3.5 × 3.5 × 4 cm (length × width × depth) filled with commercial sowing compost (Horticoop substrate, Lentse potgrond and Slingerland potgrond, Cuijk, the Netherlands) or first placed on wet filter paper in petri dishes and then transplanted to the seed trays after germination. To induce germination, seeds were stratified to break dormancy by placing the wet seeds at 4 ˚C for two to three weeks. After stratification, seeds were transferred to 20 ˚C and upon germination, plants were defined to be 0 weeks old. After approximately three weeks, when plants had developed four to five leaves, homogenously grown seedlings were selected and transplanted to individual pots of 1 L (experiment 1 & 4) or 1.35 L (experiment 2 & 3), filled with a mixture of sandy soil (70 % sand, 30 % clay) and 4 g slow release coated fertilizer (Osmocote Exact Standard, 3-4 months; Everris International, Geldermalsen, the Netherlands). Plants were grown in a greenhouse and watered regularly with tap water. Within two weeks after repotting, plants were fertilized twice with an additional 50 ml of nutrient solution (2 g L^{-1} of Kristalon, Yara International ASA, Vlaardingen, the Netherlands). The average temperature in the greenhouse was 20.1 °C and the average air humidity was 58 %.

Experimental designs

Experiment 1 Effect of decreased adventitious root formation on plant performance

We aimed to evaluate the importance of adventitious roots by experimentally manipulating the formation of these roots. Before the onset of this experiment, we first investigated the optimal methods to prevent the formation of adventitious roots in a pilot experiment (Appendix Fig. S1). Out of seven adventitious root manipulation treatments, two treatments resulted in the lowest and second lowest number of adventitious roots, respectively: a removal treatment where all the visible adventitious root primordia on the stem were removed before flooding, and a vaseline treatment where a thick layer (~ 0.5 cm) of vaseline covered the stem and prevented the stem to be in contact with the surrounding water. These two treatments were chosen for the current experiment, and compared to a control without manipulation of root formation and a control treatment where wounds similar to those in the primordium removal treatment were made to the stem tissue (leaving the

primordia intact). The latter treatment tested for potential side effects of the inevitable wounding caused by the removal treatment. All four treatments were then subjected to either partially flooded conditions, or irrigated but drained conditions, respectively, for three weeks. The latter conditions served as a control to examine the effects of flooding on plant growth.

Eighty-eight 64 (± 12.1) cm high plants were selected for homogeneity and subsequently assigned to the four treatments under both drained and flooded conditions for three weeks. Plants in drained conditions were well watered and plants in flooded conditions remained flooded to 10 cm above the soil surface throughout the experiment. During flooding, plants developed occasionally adventitious roots from root primordia covered by vaseline (on average 7 compared to 37 roots per plant in the removal treatment, and mainly only after the second week of flooding), or, more frequently, from newly formed primordia in the removal treatment. These new roots were removed every day.

Experiment 2. Nutrient uptake by adventitious roots during flooding

In this experiment, we evaluated the effect of adventitious root size on the uptake capacity of two major nutrients for which uptake the root surface may be a limiting factor, phosphorous (P) and potassium (K). Plants displaying a range of adventitious root number and length were created by subjecting 12 plants of eight weeks old to different flooding treatments. Six of the 12 plants were flooded to 15 cm above the soil surface for two weeks. The other six plants were first soil flooded (1 cm above the soil surface) for one week, and then flooded to 15 cm for another one week to have fewer and shorter adventitious roots while keeping the sediment roots flooded for the same period of time.

To be able to supply nutrient solution to the adventitious roots on the stem only, and not to the original root system in the soil, a glass cylindrical cuvette (d = 10 cm, h = 8 cm) with detachable top and bottom lids was constructed (Appendix Fig. S2). After attaching the cuvette to the stem with a clamp around the top lid, the bottom lid was sealed to the cuvette with a mixture (v:v, 1:1) of silicon sealant (Silicone Glass Sealant, Bison International B.V., Goes, the Netherlands) and corn starch (Maizena, Koopmans, Amersfoort, the Netherlands). This mixture shortens the hardening of silicon sealant to about 45 min. During this time, the floodwater was lowered to access the adventitious roots on the stem, and the adventitious roots were temporarily loosely wrapped in plastic foil after spraying with water to prevent dehydration. The cuvette was then filled with a known volume of nutrient solution (ranging between 540 to 570 ml; composition given below), the plastic foil around the

roots was removed, the cuvette closed, and the floodwater level was increased again to the level of the bottom of the cuvette.

We used rubidium (Rb), an analogue of K, to evaluate K uptake, and $H_2PO_4^-$ for P uptake. Concentrations of 17 µM Rb^+ and 50 µM $H_2PO_4^-$ in a 1/40 strength modified Hoagland solution (containing in µM: NO_3^- 440, K^+ 340, Ca^{2+} 200, Mg^{2+} 50, SO_4^{2-} 175, Na^+ 6.5, Fe^{3+} 9.0, Cl^- 5.0, BO_3^{3-} 2.5, Mn^{2+} 0.2, Zn^{2+} 0.2, Cu^{2+} 0.05, MoO_4^{2-} 0.05) were supplied to the adventitious roots. Before and after an uptake period of 6 h, a sample of 10 ml was taken from the solution in the cuvette, to determine nutrient depletion. The cuvette was then removed from the plant, and the adventitious roots were thoroughly rinsed with tap water. Subsequently, the plant was flooded in tap water for another 12 h, to determine Rb distribution over the plant tissues. The concentrations of Rb and P in the nutrient solution were measured with spectrometry (ICP-OES iCAP 6000, Thermo Fischer Scientific, Waltham MA, USA, and ICP-MS, Thermo Fisher Scientific, Waltham MA, USA). The adventitious roots were counted, and scanned using a A3-sized flatbed scanner (Epson Expression 11000XL, Japan), after which the total length, surface area and volume of the adventitious roots were calculated in WinRHIZO (Regent Instruments Inc., Québec, Canada). Rb concentrations in the plant tissues were determined by spectrometry (ICP-MS, Thermo Fisher Scientific, Waltham MA, USA) after destruction of 200 mg grinded leaf sample using a microwave oven (MLS 1200 Mega, Milestone Inc., Sorisole, Italy) (Tang *et al.* 2016). However, Rb concentrations in these samples were too low to provide reliable data, and are therefore not presented.

Experiment 3. Effects of the number of adventitious roots on leaf transpiration and photosynthesis

Eight-week-old plants were subjected to drained conditions, or to flooding. Two adventitious root manipulation treatments, with six replicates per treatment, were applied (stems covered with vaseline and stems without vaseline). Plants were well watered in the drained treatment and flooded to 10 cm above the soil surface in both flooding treatments. Plants were allowed to form adventitious roots freely in the flooding without vaseline treatment, whereas in the flooding with vaseline treatment, formation of adventitious roots was prevented by covering the basal 12 cm stem with a 0.4 mm thick layer of vaseline (Daro Vaseline, the Netherlands) prior to flooding. After three weeks, transpiration and photosynthesis were measured on a young mature leaf from each plant with a Li-Cor 6400 portable photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA). The measurements were taken at leaf temperature 23 ˚C, with saturating light at 1,000 µmol photons m^{-2} s^{-1} and 400 µmol

mol^{-1} CO_2. The temperature and relative air humidity were 20 ˚C and 60 %, respectively, throughout the experiment.

Experiment 4. Benefits of adventitious root formation under different flooding duration

To take account of the contrasting hydrological conditions which natural populations of *S. dulcamara* may experience, this experiment was performed with seeds originating from plant populations derived from a wet and a dry habitat at Texel (Chapter 2). Nine weeks after germination plants were subjected to a combination of treatments modulating the adventitious roots number and flooding duration. Plants (in six to eight replicates) were flooded to 5 cm above the soil surface either for one, two or four weeks, and subjected to root removal or control treatments. In the root removal treatment, newly formed adventitious roots (≥ 2 mm) of each plant were removed every other day. In the control treatment, adventitious roots were left intact. Plants were harvested after one, two or four weeks of flooding.

Measurements of stem height, leaf size and biomass in all experiments

Initial stem height was measured for each plant in experiment 1 before subjecting plants to the treatments. In both, experiment 1 and 4, the youngest fully expanded leaf on the main shoot was marked at the onset of the experiments. The leaf above the marked leaf was harvested one day before the final harvest to determine leaf size (LI-3100 Area Meter, Li-Cor, Lincoln, NE, USA). During harvest, stem height was measured and roots were carefully washed free of substrate. Plants were divided into leaves, stems, sediment roots and adventitious roots, of which dry weights were determined after drying the plants to constant weight at 70 ˚C in all experiments.

Data analysis

All statistical analyses were conducted in R (R Development Core Team 2014). In experiment 1, to test the effects of damage (control and vaseline *vs.* wounding and removal) and adventitious root removal (vaseline and removal *vs.* control and wounding treatments) on total biomass, stem height and leaf size, a two-way ANCOVA (type III) with damage and adventitious root removal as main effects was conducted using the *car* package. Initial stem height was treated as a covariate for total biomass and stem height analysis. Separate one-way ANOVAs were then conducted to test the effects of the four different treatments (control, wounding, vaseline and removal)

on total biomass, stem height and leaf size under both flooding and drained conditions. The total biomass and initial stem height were ln-transformed to increase the homogeneity of the variance. In experiment 2, linear regressions were performed to test the relationship between nutrient uptake rate and the surface area of the adventitious roots. In experiment 3, separate one-way ANOVAs (type III) were performed on plants in the flooding without vaseline and flooding with vaseline treatments to test the effect of the presence of adventitious roots on leaf transpiration and photosynthesis. To test the effect of flooding on these two parameters, one-way ANOVAs (type III) were then performed on plants in the control and flooding without vaseline treatments. The significance levels were adjusted from 0.01, 0.05 and 0.1 to 0.005, 0.025 and 0.05, respectively, to account for multiple comparisons. In experiment 4, the main effects of adventitious roots (with adventitious roots *vs.* without adventitious roots), flooding duration (one, two and four weeks flooding) and habitat (dry *vs.* wet), and their interactions were analysed in a three-way ANOVA (type III). Within each habitat, the effect of flooding duration on the adventitious root biomass of plants with intact adventitious roots was analysed in one-way ANOVA (type III) followed by post hoc comparisons using the *glht* function under the *multcomp* package.

Results

Decreased numbers of adventitious roots negatively affected plant growth during flooding

To be able to comprehensively understand the importance of adventitious roots, preventing their formation provides an opportunity to evaluate whether negative effects occur when plants are not able to develop these roots during flooding. Plant performance in terms of biomass gain, plant size and leaf expansion were significantly decreased (25 %, 18 % and 58 %, respectively) by removal of the adventitious root primordia or vaseline application on the stem (Fig. 2 a, b & c, Table 1) prior to flooded conditions. Damaging the stem in a similar way but leaving the primorida and root outgrowth intact (wounding treatment) had no effect on the final total biomass, stem height and leaf size under flooded conditions (Fig. 2, Table 1), indicating that the negative effects on plant growth caused by adventitious root removal were not due to damage but due to the lack of adventitious roots. None of the three treatments (vaseline, removal and wounding) affected total biomass, stem height and leaf size of plants under drained conditions (Fig. 2), suggesting that these treatments did not limit plant growth *per se*.

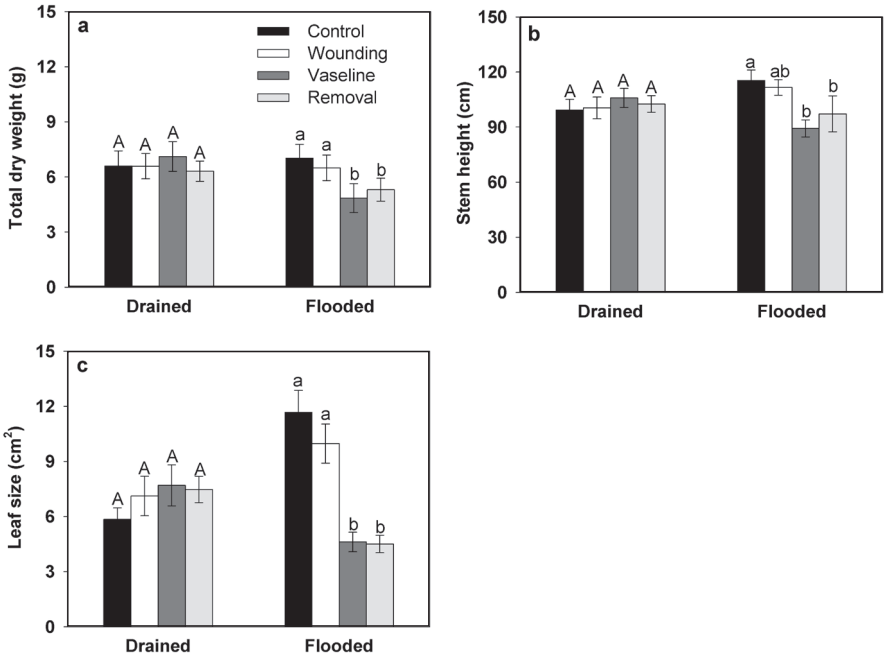

Figure 2. Mean (± 1 SE) total plant dry weight (a), stem height (b) and leaf size (c) after three weeks of drained or partially flooded conditions, and control treatment (no primordia removal), vaseline treatment (where the lower stem parts including the root primordia were covered with vaseline), removal of adventitious root primordia, or wounding treatment (where similar wounds as with primordia removal were made on the stem without damaging the primordia), respectively. Significant differences within drained or flooded conditions are indicated with different letters ($p < 0.05$, $n = 11$).

Table 1 Two-way ANCOVA results on total dry weight, stem height and leaf size with adventitious root (AR) removal (vaseline and removal treatments *vs.* intact and wounding treatments) and damage (vaseline and intact treatments *vs.* removal and wounding treatments) as main effects. For the analysis of total dry weight and stem height, initial stem height was included as a covariate.

		F-values		
	d.f.	**Total dry weight**	**Stem height**	**Leaf size**
AR removal	1	**18.153*****	**15.387*****	**64.075*****
Damage	2	0.705	0.157	0.695
Initial stem height	1	**243.972*****	**26.944*****	------
AR removal × Damage	1	1.397	1.014	0.570

Total dry weight was ln-transformed to meet the assumption of ANOVA. The significances are *** $p < 0.001$, ** $p < 0.01$. Values in bold indicate significant results.

Adventitious roots contributed to nutrient and water uptake

To reveal potential mechanisms contributing to the beneficial role of adventitious roots during flooding, we investigated the main function of these roots by evaluating nutrient uptake, and by estimating water status of the plant via transpiration measurements. After 6 h incubation time, both P and Rb, as a substitute for K, were shown to have been taken up by the adventitious roots at rates that ranged from 0.11 to 1.95 μmol h^{-1} and 0.05 to 0.54 μmol h^{-1} for P and Rb, respectively, depending on root size (Fig. 3). The uptake rates of both P and Rb strongly correlated with the total surface area of the adventitious roots (Fig. 3), suggesting that this surface determines ion uptake during flooding. Although just marginally significant due to relatively large variation among plants, transpiration and photsynthesis rates were on average respectively 45 % and 25 % lower in plants without adventitious roots (flooding with vaseline treatment), compared to plants with adventitious roots (flooding without vaseline treatment), while the latter group had similar transpiration and photosynthesis rates as the control plants (Fig.4, Table 3).

The effect of removing adventitious roots on plant growth was dependent on flooding duration

The size of the adventitious root system was significantly determined by flooding duration (Fig.5), because elongation and branching of the adventitious roots, rather than the fast initiation of primordia outgrowth, were primarily determining root system size. Since nutrient uptake significantly correlated with the size of the adventitious root system (Fig. 3), the potential benefits of developing adventitious roots were also expected to depend on flooding duration. To confirm this hypothesis, we conducted an experiment investigating the effects of adventitious root removal in plants subjected to different flooding durations. Removing the adventitious roots significantly decreased biomass accumulation, leaf size and the size of the plants subjected to four weeks flooding, but not to one or two weeks flooding (Fig. 6, Table 2), affirming a dependence of plant performance on adventitious roots when these have grown out to a sufficient size. Plants originating from both dry and wet habitats showed similar responses to adventitious root removal in terms of biomass gain and final height (Fig. 6, Table 2).

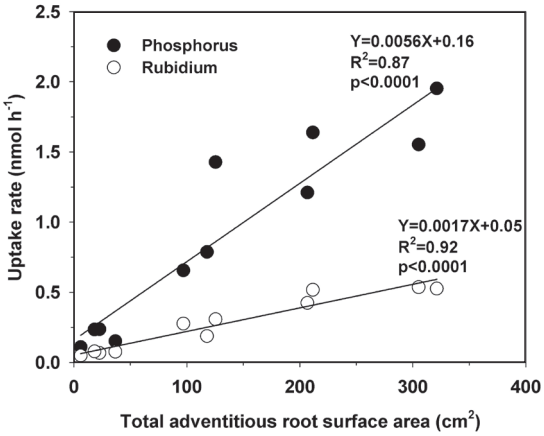

Figure 3. Correlation between total surface area of the adventitious roots of *S. dulcamara* and the uptake rates of phosphorus and rubidium, respectively, under partially flooded conditions. The uptake rates were calculated after 6 h of incubation of the adventitious roots in a closed cuvette that was fixed around the stem and contained nutrient solution. Slopes, correlation coefficients and statistical significance of the linear regression lines are indicated.

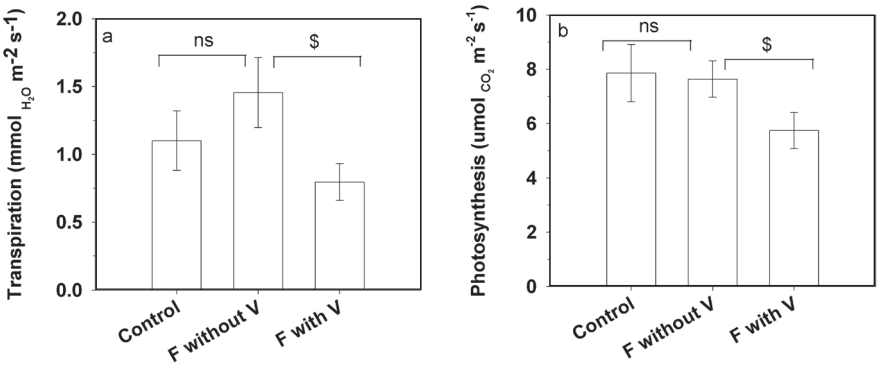

Figure 4. Mean (± 1 SE) rates of leaf transpiration (a) and photosynthesis (b) of plants in drained control (Control; plants were grown under drained conditions), flooding (F without V; plants were partially flooded) and vaseline (F with V; plants were partially flooded and vaseline was covered on the flooded stem) treatments. The significance levels were adjusted from 0.01, 0.05 and 0.1 to 0.005, 0.025 and 0.05, respectively, to take account of multiple comparisons, $ $0.025 < p < 0.05$, ns $p > 0.05$.

Table 2 The results of three-way ANOVA on total dry weight, stem height and leaf size with adventitious root treatment (with ARs *vs.* without ARs), flooding duration (one, two and four weeks) and habitat (dry *vs.* wet) as main effects.

		F-values		
	d.f.	**Total dry mass**	**Stem height**	**Leaf size**
AR treatment (A)	1	**19.993*****	**27.410*****	**62.373*****
Duration (D)	2	**51.428*****	**70.716*****	**15.240*****
Habitat (H)	1	**40.476*****	**15.965*****	0.803
A × D	2	**15.663*****	**9.721*****	**23.141*****
A × H	1	0.279	0.051	0.063
D × H	2	**5.885****	0.249	0.318
A × D × H	2	1.031	0.490	**2.534**$^{\$}$

Leaf size was ln-transformed to meet the assumption of ANOVA. The significances are *** p < 0.001, ** p < 0.01, * p < 0.05, $ p < 0.1. Values in bold indicate (marginally) significant results.

Table 3 The results of one-way ANOVA on the effect of adventitious root (AR) presence and flooding on transpiration and photosynthesis. The significance levels were adjusted from 0.01, 0.05 and 0.1 to 0.005, 0.025 and 0.05 to take account of multiple comparisons.

		F-values	
	d.f.	**Transpiration**	**Photosynthesis**
AR presence	1	**5.126**$^{\$}$	**4.028**$^{\$}$
Flooding	1	1.098	0.031

$ denotes 0.025 < p < 0.05. Values in bold indicate marginally significant results.

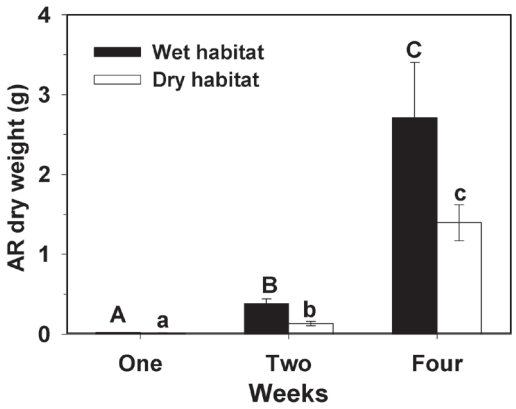

Figure 5. Mean (± 1 SE) adventitious root biomass of plants originating from dry and wet habitats after one, two and four weeks of partial submergence, respectively. Significant differences within each habitat type are indicated with different letters (p < 0.05, n = 6 to 8).

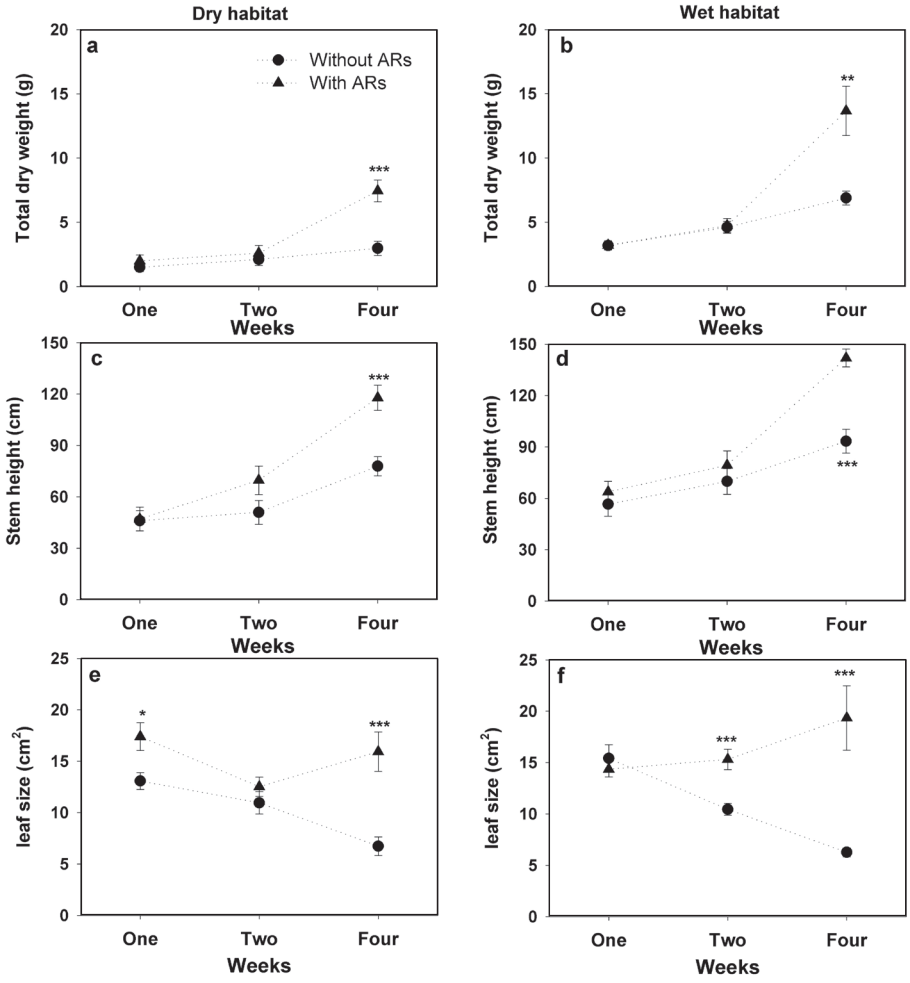

Figure 6. Mean (± 1 SE) total dry weight (a, b), stem height (c, d) and leaf size (e, f) for plants originating from dry (a, c, e) and wet habitats (b, d, f) with adventitious roots (ARs) and without ARs after one, two and four weeks of flooding, respectively. Significant effects of adventitious root removal within each flooding duration are indicated by asterisks (**), *** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$, $ $0.05 < p < 0.1$.

Discussion

Flooding regimes in natural habitats often are unpredictable, as they may vary both in time and space (Blom *et al.* 1990; van der Sman, Joosten & Blom 1993). This may affect the selection on the adaptive traits that are essential to buffer the negative effects of flooding on plant growth (Setter & Laureles 1996; Chen *et al.* 2011). If such traits are constitutively present in a flood-prone plant population, they may incur costs if flooding does not occur permanently. However, also flooding-induced traits may not be beneficial if the duration of flooding is too short. In this study, we quantified the contribution of flooding-induced adventitious root formation to plant growth and subsequently investigated whether this contribution depends on flooding duration. Our results revealed a positive effect of adventitious roots on plant performance, with respect to transpiration, photosynthesis and biomass accumulation, in long-term flooding but not in short-term flooding. Regarding the function of adventitious roots, we found a clear positive correlation between nutrient uptake and the size of the adventitious root system. This relationship, possibly also valid for water uptake, may be the underlying reason why adventitious roots were only being advantageous under long flooding duration. This may be due to the adventitious roots only gradually taking over the function of the sediment roots, as their biomass relative to the sediment roots increased exponentially, from less than 10 % after one week of flooding, to 600 -700 % after four weeks of flooding. This implies that there is a time-lag between the onset of flooding and sustained root function. In addition, adventitious roots that grow in the floodwater mostly dry out and die upon subsidence of the flood. Our data show that adventitious roots convey benefits under long-term, but not under short-term flooding, corroborating our hypothesis. However, in contrast to our expectation, no clear costs in terms of energy investment into adventitious root production and maintenance were detected under short-term flooding, which may explain why the ability to produce adventitous roots is maintained over a wide range of habitats.

Adventitious roots confer fitness advantages in long-term but not in short-term flooding

As hypothesized, our experiment revealed a net benefit mediated by adventitious roots in terms of increased biomass accumulation only for long-term flooding (three and four weeks of flooding in the present study), but not for short-term flooding (less than two weeks of flooding in the present study). This observation is likely explained by the increased size of the

adventitious root system with longer flooding duration. The formation of the first adventitious roots in *S. dulcamara* commences very quickly, usually within two days (Dawood *et al.* 2014; Zhang *et al.* 2015). However, a relatively large adventitious root system characterized by the presence of lateral roots and a large surface area needs significantly longer time to develop (Q. Zhang, personal observation). Within the first two weeks of flooding, the adventitious roots are unbranched and only constitutes approximately 0.5 % (one week) to 1.2 % (two weeks) of the total plant dry weight, which is more than 10-fold smaller than the biomass of adventitous roots produced during longer flooding duration (11 % of total plant weight after flooding lasting for three weeks and 20 % after four weeks). Roots of this size substantially contribute to resource acquisition, and consequently in their contribution to plant growth. The beneficial effects of adventitious roots have also been shown in previous studies in several other species during relatively long-lasting flooding events, varying from 15 to 110 days. Tsukahara and Kozlowski (1985) found that removing the adventitious roots of seedlings of the tree species *Platanus occidentalis* significantly reduced leaf initiation and expansion, growth rates and dry weight. Also, adventitious root removal resulted in siginifcantly lower dry weight of the legumes *Macroptilium lathyroides* (16 % reduction) and *Vigna luteola* (26 % reduction) (Javier 1987). However, as in these studies the effects of adventitious roots on plant growth was only studied at one time point, they did not provide information about the production and potential benefits of adventitous roots during the course of flooding events.

As plastic responses are usually not only associated with benefits but also with costs in terms of energy investment into, e.g., production and maintenance of the plastic response (DeWitt, Sih & Wilson 1998; Dorn, Pyle & Schmitt 2000; Huber *et al.* 2004; Weijschedé *et al.* 2006; Murren *et al.* 2015), it can be expected that plants may incur costs from adventitious root formation, especially when flooding lasts only for a few days. However, in contrast to our hyphothesis, plants that invested into adventitous root development attained the same biomass as plants whose adventitious roots were removed immediately after emergence from the stem during short-term flooding. This implies that the costs of investing into the growth and maintenance of these still small adventitious roots are relatively low, possibly also because these roots contain highly porous aerenchyma, which is known to reduce respiration and cheaper to maintain than regular root tissues (Fan *et al.* 2003; Postma & Lynch 2011). Such presumably low costs of adventitious root production may contribute to the lack of habitat effect on adventitious root formation in *S. dulcamara,* such as found in a previous study (Chapter 2), where the potential

to produce adventitious roots is even maintained in dry populations which virtually never experience flooding (Chapter 2).

Adventitious roots maintain nutrient and water uptake during flooding

Adventitious roots showed to be beneficial in flooding that lasted for long periods, but to unravel the mechanism underlying this benefical role, further studies into the function of these roots were needed. Soil rapidly becomes anoxic upon flooding, causing nutrient availability in the soil to decrease (Kozlowski & Pallardy 1984). In addition, the lack of oxygen may cause decay of the primary root system (Visser *et al.* 2015). Such lower nutrient availability together with impeded functioning of the primary roots may lead to nutrient deficiency in the entire plant (Drew & Sisworo 1979; Trought & Drew 1980). It has been suggested that the adventitious roots may take up nutrients and water from the floodwater, thereby partly buffering the reduced functionality of the root system produced prior to flooding and which is not adapted to flooding conditions (Jackson 1955; Javier 1987; Sauter 2013). In the present study, we quantified the uptake capacity of two major elements P and K (the latter by its analogue Rb) by the adventitious roots of *S. dulcamara* during flooding. The adventitious roots showed considerable P and Rb acquisition, as 4 -73 % and 3 - 36 % of P and Rb in the cuvette were taken up within 6 h, respectively, depending on the surface area of adventitious roots. Other species such as deepwater rice have also been shown to take up considerable amounts of ^{15}N through the nodal adventitious roots (roots generated from the stem nodes) during flooding (Khan, Ventura & Vergara 1982). In wheat, the nodal adventitious roots contributed equally to nitrate and potassium uptake in O_2-deficient media compared to the sediment root system (Kuiper, Walton & Greenway 1994). Being able to take up nutrients through the adventitious roots from the floodwater can thus substantially improve plant survival and performance during flooding and be the major mechanism responsibe for increased plant performance upon adventitious root formation.

In addition to nutrient uptake, adventitious roots may also contribute to water uptake during flooding (Calvo-Polanco, Senorans & Zwiazek 2012). Flooding causes stomata closure, following a reduced water uptake of the flooded plant (Kozlowski & Pallardy 1984; Else *et al.* 2009). In our study, plants with intact adventitious roots had twice the transpiration rates of plants that were prevented from producing adventitious roots during flooding, suggesting that adventitious roots did increase water status in flooded plants. Similar results were found in a woody species *Fraxinus pennsylvanica* that plants maintained

higher transpiration rates in the presence of adventitious roots (Gomes & Kozlowski 1980). Intertestingly, in the present study, while adventitious roots gradually took over the function of the original sediment roots, flooded plants still remained constant sediment root biomass (unpublished data) during flooding. The survival of sediment roots may suggest that in addition to anchoring, the sediment roots may still be functioning. These sediment roots are potentially essential for the continuation of plant growth after the waterlevel decreases again. However, further studies focusing on the relative contribution of adventitious roots and sediment roots to resource acquisition are required to assess the relative importance of each of the two root systems during flooding. The mechanism about how these sediment roots survive in hypoxic conditions may also deserve more attention.

Conclusions

We comprehensively demonstrated in this study the importance of adventitious roots during partial submergence, the underlying mechanisms of the benefits associated with the adventitious roots, and the benefits and/or costs of these roots under different flooding regimes. Adventitious roots of *S. dulcamara* plants were capable of taking up nutrients and water from the floodwater, thus conferring benefits to plant performance. However, as the nutrient uptake was strongly affected by the size of the adventitious root system, the fitness advantages were only pronounced in long-term flooding, after a sizeable adventitious root system had developed. This fitness advantage associated with adventitious root production may provide a potential for selection on adventitious root formation in environments with predictable long-lasting flooding events. In contrast to our expectation, the adventitious roots did not confer apparent costs when flooding lasted for a short period of time, suggesting that adventitious root formation is not a very costly process. This makes it likely that species can maintain this plasticity in unpredictable environments. This notion was confirmed in earlier work on this species, where populations from dry habitats that had never experienced flooding, retained the same potential for adventitious root development as populations originating from wet habitats (Chapter 2). Such constitutive plasticity may enable this species to occur in the contrasting hydrological environments without evolving specific adaptation to the local environment (Chapter 2).

Appendix

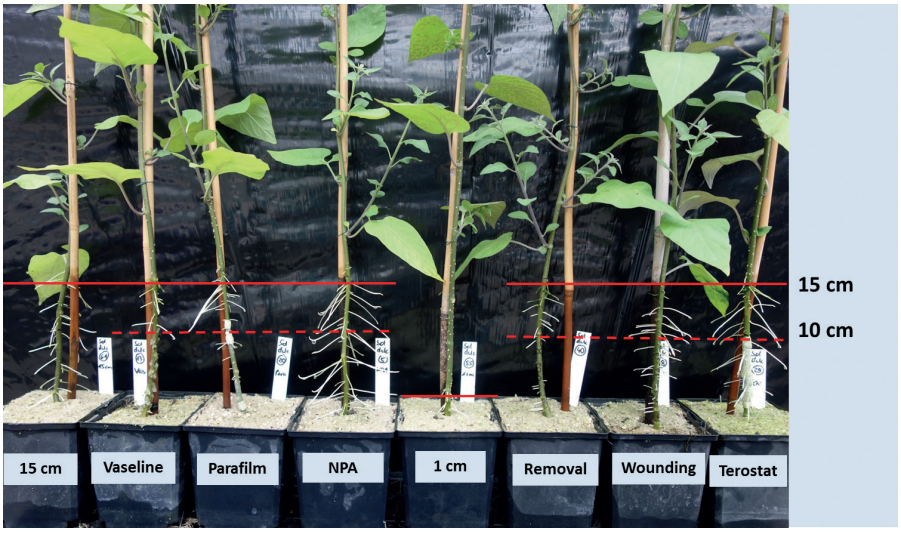

Figure S1. Different methods to prevent the adventitious root formation. The solid lines indicate the water levels (1 cm and 15 cm), the dashed lines indicate the height of the treatments that prevent adventitious root formation. The treatment "15 cm" was treated as a control where plants were flooded to 15 cm above the soil surface. In the vaseline treatment, vaseline was applied to cover all the visible adventitious root primordia to create a barrier between the root primordia and the surrounding water. In the parafilm and terostat treatments, the stem was wrapped either with parafilm and terostat (a vehicle body sealant; Teroson, Henkel AG & Co. KGaA-40191 Dusseldorf, Germany) together (parafilm treatment) or with terostat alone (terostat treatment), respectively. In the NPA treatment, a 2 mm circle of N-1-Naphthylphthalamic acid (NPA, 10 mM) dissolved in lanolin was applied to the stem at 1 cm above the water level. In "1 cm" treatment, plants were raised to lower down the floodwater from 15 cm to 1 cm to reduce the contact area of the root primordia with water. The removal treatment was achieved by cutting off all the visible root primordia on the stem, and in the wounding treatment, the same wounds were created imitating the damage from removal treatment on the non-primordium part of the stem. Vaseline, removal and wounding treatments were used in experiment 1.

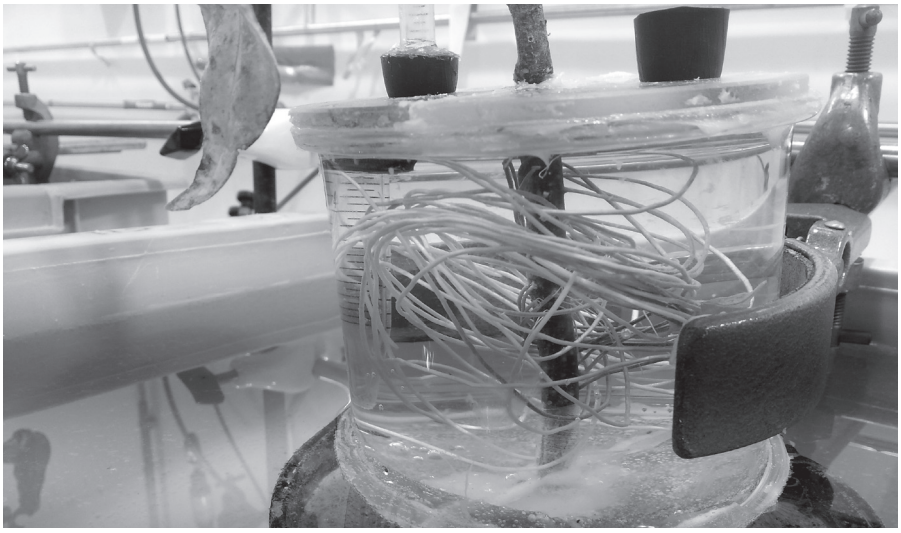

Figure S2. The cuvette used for nutrient uptake (P and Rb) by adventitious roots of *S. dulcamara* in experiment 2. The adventitious roots were sealed in the cuvette filled with nutrient solution for 6 h.

Chapter 5

Factors affecting adventitious root formation during complete submergence of *Solanum dulcamara*

5

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Manuscript in preparation

Abstract

Flooding is a compound stress that imposes a number of limitations to plant development. Different adaptive traits have evolved in plants to cope with such hostile conditions. However, when a plant is completely submerged, the expression of adaptive traits may be constrained. We aimed to unravel the mechanisms constraining adaptive adventitious root formation in *Solanum dulcamara* during complete submergence. After becoming completely submerged, plants face decreased availability of multiple resources such as oxygen, carbon dioxide and light, which inevitably causes carbohydrate deficiency as well as leading to reduced transport of essential hormones such as auxin. We investigated the effects of oxygen concentration, internally stored and newly produced carbohydrates and manipulation of auxin concentrations on the formation of adventitious roots. We found that all three factors may significantly influence adventitious root formation during complete submergence, but their effect sizes depended on the timing and location of oxygen and carbohydrate supply. Access to atmospheric oxygen was more important for adventitious root formation than the concentration of dissolved oxygen in the floodwater, and newly formed carbohydrates through ongoing photosynthesis rather than stored carbohydrates fuelled adventitious root formation during flooding. External auxin application significantly stimulated the formation of adventitious roots, which indicates that a sub-optimal auxin concentration may have suppressed this adaptive response during complete submergence. The results suggest that the impact of a stress factor such as flooding on plant response may strongly depend on the interplay of various factors. Linking these results to the distribution of *S. dulcamara* along the hydrological gradient in the field, the lack of adventitious root formation during complete submergence may prevent this species to persist in areas that are often subjected to prolonged deep flooding.

Introduction

Soil flooding and partial flooding impose severe hypoxia stress on terrestrial plants, causing a malfunctioning of the belowground root system and ultimately plant death (Armstrong & Drew 2002; Sauter 2013). Complete submergence of the shoot aggravates the stress by challenging the plants with further limited access to oxygen and additional stresses, such as a substantial light and CO_2 reduction (Voesenek *et al.* 2006; Manzur *et al.* 2009). Due to such additional constraints, the acclimations plants have evolved to cope with partial flooding may be restrained during complete submergence, resulting in a decreased flooding resistance. Previous studies revealed that adventitious root formation, an important adaptive response that alleviates oxygen deficiency and contributes to resource uptake during partial flooding (Visser *et al.* 1996; Rich, Ludwig & Colmer 2012; Sauter 2013; Chapter 4), was suppressed in complete submergence (van der Sman, Blom & Barendse 1993; Zhang *et al.* 2015). While the mechanisms regulating adventitious root formation are well studied (Negi *et al.* 2010; Dawood *et al.* 2014; Dawood *et al.* 2016; Steffens & Rasmussen 2016), much less is known about the mechanisms that constrain this important adaptive response.

The most apparent difference between partial and complete submergence may be the access to the atmosphere that significantly increases oxygen availability to the plant. Previous studies observed that after the shoot regained contact with air the inhibitory effect of complete submergence on adventitious root formation was released (van der Sman, Blom & Barendse 1993; Zhang *et al.* 2015), suggesting that oxygen might be a key factor explaining the lack of adventitious root formation during complete submergence. Oxygen is essential for root growth and functioning (Colmer & Greenway 2011), and low internal oxygen concentrations caused by flooding inhibit aerobic respiration and hence energy production (Bailey-Serres, Lee & Brinton 2012). Highly energy demanding processes involved in root initiation and elongation, i.e., cell division and expansion, are then likely to cease due to lack of energy (Perata, Guglielminetti & Alpi 1997).

Complete submergence may also influence the availability of carbohydrates that are the energy source essential for plant functioning, and, hence, acclimation to flooding stress (Mommer & Visser 2005). Limited oxygen availability may hamper the release of free hexoses from stored polycarbohydrates due to impeded aerobic respiration (Voesenek *et al.* 2006), thus restraining the available carbon for energy production and building root biomass. Furthermore, the production of new carbohydrates may be restricted

as the availability of CO_2 and light is strongly reduced under complete submergence, contributing to a decreased photosynthesis (Nielsen 1993; Voesenek *et al.* 1993; Mommer, Pons & Visser 2006). Therefore, an inadequate supply of carbohydrates might constrain the development of flooding acclimations, similar to its effect on flooding-induced petiole elongation in *Rumex palustris* (Huber *et al.* 2012). Reducing carbohydrate levels by depletion and/or through removing mature leaves has been shown to substantially limit this elongation response (Groeneveld & Voesenek 2003). Adventitious root formation is potentially an even more energy demanding process, consisting of both cell elongation and division (Dawood *et al.* 2014) rather than only cell elongation as in petiole elongation. Also in plant cuttings, adventitious root development of *Pelargonium* was positively correlated with carbohydrate content (Druege, Zerche & Kadner 2004), and additional carbohydrate supply enhanced the adventitious rooting capacity in cuttings of *Eucalyptus saligna* and *E. globulus* (Corrêa *et al.* 2005). This correlation between carbohydrate supply and the magnitude of adventitious rooting suggests that the availability of carbohydrates is essential for the actual flooding acclimation to realize.

The formation of adventitious roots in response to partial flooding is regulated by phytohormones: two positive regulators, i.e., ethylene and auxin, and one negative regulator, i.e., abscisic acid (ABA) (Dawood *et al.* 2016; Steffens & Rasmussen 2016). Auxin in particular is transported in a polar, basipetal manner through a group of PIN proteins (Jones 1998; Vieten *et al.* 2007). Its basipetal polar transport is required for the adventitious root formation in various species (for rice: Zhou *et al.* 2003; Xu *et al.* 2005; for tomato: Negi *et al.* 2010; Vidoz *et al.* 2010; for *Solanum dulcamara*: Dawood *et al.* 2016). As the polar auxin transport is an energy-demanding process, it can be inhibited under anaerobic conditions (Wilkins & Martin 1967). In addition, auxin production in the shoot may be negatively influenced due to the slowing-down of metabolism during complete submergence, potentially resulting in reduced auxin availability. The subsequent auxin limitation in the stem may lead to the lack of activation of the dormant adventitious root primordia during complete submergence.

This manuscript aims to explore which of the above mentioned factors contributes to the inhibition of adventitious root formation by complete submergence. More specifically, we addressed the following questions: 1) Does increased oxygen concentration in the floodwater enhance adventitious root formation during complete submergence? If so, how strong is this effect as compared to partial flooding, where the contact of the shoot with the atmosphere is maintained? 2) Does adventitious root formation depend

on endogenous carbohydrate content? If so, are the stored carbohydrates or rather the carbohydrates formed after the onset of flooding controlling adventitious root formation? 3) Does auxin limitation interrupt the hormonal pathway regulating the activation of adventitious root primordia? If so, will releasing this limitation by auxin addition lead to a rescue of the adventitious root outgrowth?

Material and Methods

Plant material

Plant material for the oxygen and carbohydrate experiments

Seeds were collected from plants at the shore of a freshwater dune lake at the North Sea island Texel (53° 7′ 24″ N., 4° 47′ 10″ E) in the Netherlands in September 2013. The seeds were cleaned, dried and then stored in a dark cold chamber at 4°C. On 8 October 2014, 300 seeds were surface-sterilized with 15 % (w:v) trisodium phosphate in water and subsequently rinsed with abundant tap water. After sterilization, seeds were sown on wet filter paper in petri dishes, each containing 3 ml 10^{-5} M gibberellin solution to break seed dormancy and promote germination. The seeds were kept at 20 ˚C for germination during two weeks. Germinated seeds were transplanted into seed trays (cell dimensions 3.5 × 3.5 × 4 cm, length × width × depth) filled with commercial soil compost (Horticoop substrate, Lentse potgrond & Slingerland potgrond, Cuijk, the Netherlands) and placed in the greenhouse. After three weeks, 140 seedlings were transplanted into individual 1.3 L pots, filled with a 70 % sand : 30 % clay mixture (v:v) amended with 4 g L^{-1} slow release fertilizer (Osmocote® Exact Standard, NPK 15-9-12+2MgO+tracing elements, release time 5-6 months, Everris International B.V., Geldermalsen, the Netherlands). These plants were used in the "oxygen experiment". The remaining 160 seedlings were transplanted into individual 0.9-L pots filled with the same soil as mentioned above. These plants were used in the "carbohydrate experiment". Within the first two weeks after transplanting, up to 120 mL nutrient solution (2 g L^{-1} Kristalon in rainwater, Yara International ASA, Vlaardingen, the Netherlands) in total was added to each pot at two different time points to prevent nutrient limitation at the start of the experiment. Plants were regularly watered with rainwater and grown under ambient greenhouse conditions: the temperature was on average 21.5 °C during the day (06:00-22:00 h) and 17.2 °C during night time.

Plant material for the auxin experiment

Seeds from the same seed batch as in the oxygen and carbohydrate experiments were used to investigate the effect of auxin supply on adventitious root formation under complete submergence. Fifty seeds were sown on 13 March 2015 on top of glass beads in water after being surface-sterilized as described above, and subsequently kept at 4 °C to break dormancy. The seeds were subsequently transferred to 20 °C for germination after three weeks of stratification. After germination, 30 equal-sized seedlings were transplanted into seed trays (cell dimensions 3.5 × 3.5 × 4 cm, length × width × depth) in the third week after stratification. After another week, all seedlings were transplanted into 1.3-L individual pots filled with the same soil as above, but with 4 g L^{-1} slow-release fertilizer Osmocote. Within the first week after transplanting, 60 mL nutrient solution (2 g L^{-1} of Kristalon in rainwater; Yara International ASA; Vlaardingen; The Netherlands) were added to each pot to avoid nutrient limitation. Plants were watered regularly with rainwater and kept in the greenhouse until being subjected to the treatments.

Experimental set-ups

Oxygen experiments

Previous research showed that *S. dulcamara* plants only form adventitious roots when partially flooded but not when completely submerged (Zhang *et al.* 2015). Since complete submergence causes more severe oxygen shortage than partial flooding, resource limitation in terms of oxygen shortage may suppress the growth of adventitious roots. To test this, two experiments were performed sequentially, in a growth room at a light intensity of 260 µmol m^{-2} s^{-1} PAR, 22 °C and a 12 h photoperiod.

Oxygen enrichment during complete submergence

This experiment aimed to test whether an increased oxygen concentration in the floodwater promotes adventitious root formation under complete submergence. Two oxygen concentrations in tapwater, 21 kPa (the ambient concentration in air) and 42 kPa (two fold the concentration in air), were achieved by pumping either atmospheric air or a mixture of air and pure oxygen through the water column with bubble stones, at a rate of 10 L min^{-1} through a gas flow controller (El-flow® Select mass flow controller, Bronckhorst® High Tech Holland, Veenendaal, the Netherlands; software: FlowView, version 1.23). For each oxygen concentration, 12 plants of seven and half weeks old were fully submerged in six transparent glass aquaria (31 × 31 × 60 cm^3; two plants per aquarium) and three plants in another three transparent glass aquaria (20 ×

20 × 60 cm^3; one plant per aquarium). A firm net covered with a plastic foil was placed at the water surface of each aquarium to prevent gas exchange with the air and to prevent the plants from growing out of the water. As previous experiments showed that adventitious roots typically started emerging after 2 days and the number of newly developing roots levelled off after one week, the present experiment lasted for seven days, throughout which water was replenished on a daily basis.

Oxygen depletion during partial flooding

This experiment was performed to test whether a low oxygen concentration in the floodwater delays adventitious root formation in partially flooded plants. Twenty-four nine-week-old *S. dulcamara* plants were flooded to 7 cm above the soil surface in 12 containers of 29.5 x 39.5 x 20 cm^3, with one half of the containers subjected to 1 kPa and the other half to 21 kPa dissolved oxygen in the floodwater, by pumping either pure nitrogen gas or air into the tap water with bubble stones through a gas flow controller at 10 L min^{-1} (El-flow® Select mass flow controller, Bronckhorst® High Tech Holland, Veenendaal, the Netherlands; software: FlowView, version 1.23). This experiment lasted for seven days.

Measurements

For both experiments, the oxygen concentrations in the water were determined daily with an oxygen optode sensor (Fibox 3 LCD trace, Precision Sensing GmbH, Regensburg, Germany). The number of adventitious roots was counted and the longest adventitious root was measured every day. At harvest, the total length of the adventitious roots per plant was determined using a flatbed root scanner (Expression 11000XL, Epson, Japan; software: WinRHIZO 2005, Regent Instruments Inc., Québec, Canada). The dry weight of the adventitious roots was determined after oven-drying at 70 °C for 48 h.

Auxin experiment

Six plants were subjected to an auxin and a control treatment, respectively. The natural and most commonly found form of auxin is indole-3-acetic acid (IAA), but since this compound is quite unstable upon application, the synthetic analogue 1-naphthaleneacetic acid (NAA) was used in a concentration of $3.3*10^{-2}$ M (dissolved in 10 mM NaOH). This concentration was chosen because it led to the strongest adventitious root formation in a pilot experiment (data not shown).

In 12 plants side branches on the basal 15 cm stem were pruned a week before the experiment to facilitate attachment of a glass cuvette of 5 mL around the stem. The primordia that were enclosed in the cuvette were counted and marked to determine the percentage of root emergence after treatments. After injecting NAA solution (auxin treatment) or distilled water (control treatment) into the cuvettes, plants were completely submerged in 12 glass aquaria of 20 × 20 × 60 cm^3 for ten days, with air provided by pumps through bubble stones through a gas flow controller at 10 L min^{-1} (El-flow® Select mass flow controller, Bronckhorst® High Tech Holland, Veenendaal, the Netherlands; software: FlowView, version 1.23). The plants were kept submerged by mounting chicken wire together with transparent plastic foil 1 cm below the water surface. At harvest, the number of adventitious roots (> 1 mm) was counted, and the length of these roots was measured.

Carbohydrate experiment

In this experiment, the carbohydrate level of the plants was manipulated to test whether access to carbohydrates is essential for adventitious root formation. As plants contain stored carbohydrates, but also continue to form new carbohydrates via photosynthesis, we aimed to disentangle the effect of these stored and newly formed carbohydrates on adventitious root formation. Stored carbohydrates were experimentally depleted by a pretreatment in which 30 homogeneous plants of eight weeks old were kept in dark for 48 h. To prevent induction of the shade avoidance syndrome (e.g., shoot elongation) in these plants, a low amount of red light (intensity 1.0 μE m^{-2} s^{-1}; red:far-red ratio of 6.6 and insufficiently strong to stimulate photosynthesis) was supplied by filtering the light of a fluorescent lamp (Philips; 2 x TL 20W) through a transparent plastic red filter (Groeneveld & Voesenek 2003). As a control, another 30 homogeneous plants were kept under ambient greenhouse conditions for 48 h (supplemental 1000 W High Pressure Sodium lamps when irradiance was less than 185 W m^{-2} during the day (06:00-22:00 h)). After carbohydrate depletion, 10 plants from each pre-treatment were harvested for determination of the non-structural carbohydrate concentration, and 20 plants from each pre-treatment were equally divided over a shade and a non-shade treatment, resulting in four different groups of plants, namely dark-no shade, dark-shade, light-no shade and light-shade. Plants subjected to shade conditions were grown in shade cages covered with one layer of transparent green plastic (type 122, fern green, LEE filters, Andover, UK), which reduced light availability to 30 % and red:far-red ratio to 0.3. Plants subjected to no-shade conditions were grown in cages covered with transparent clear plastic

(type 130, clear, LEE filters, Andover, UK), which reduced light availability by 2 % and did not affect spectral composition of the incident radiation. Each cage contained five containers with two plants from each pre-treatment in the same container (i.e., four plants per container). All plants were constantly flooded to 8 cm above the soil surface for seven days. The average temperature during the experiment was on average 21.5 °C during the day (06:00-22:00 h) and 17.2 °C during night time.

Measurements

The number of adventitious roots was counted and the longest adventitious root was measured with a ruler under water every day during flooding. At harvest, all the adventitious roots (> 1 mm) were collected and total root length and biomass were determined as described above.

The total amount of carbohydrates was measured as the total amount of non-structural hexoses including starch, glucose and fructose. To determine the effect of depletion of carbohydrates in the depletion pre-treatments, for each plant, five fully expanded leaves (leaf 7^{th} to 11^{th}, counted from the shoot apex) and the cortical basal stem tissue (length 8 cm; measured from 2 cm above the soil surface) were taken immediately after the depletion period. At final harvest, one mature leaf (6^{th} leaf, counted from the shoot apex) and the cortical basal stem tissue (see above) from each plant was used to determine the effects of shading during the flooding treatment on carbohydrate concentrations.

Hexose concentrations were determined following the protocol described in Chen *et al.* (2010). Leaves and cortical stem tissues were harvested, immediately frozen in liquid nitrogen, and subsequently freeze-dried for 48 h. After freeze-drying, each leaf and cortical stem sample was ground into homogeneous powder, of which 10 mg was boiled with 3 ml 2.7 M HCl for one hour to extract free hexoses from starch. The extraction was neutralized with 2.7 ml 3 M NaOH and subsequently centrifuged (352 g) for ten minutes.

The concentration of carbohydrates in the extracts was determined by using an anthrone color reaction (Yemm & Willis 1954). The anthrone reagent was prepared in advance by slowly adding 400 mg anthrone dissolved in 200 mL H_2SO_4 (98 %) to a mixture of 60 mL demi water and 15 mL 95 % (v:v) ethanol in a conical flask on ice. This reagent (2.5 ml; containing 0.04 mM anthrone, 6 % (v:v) ethanol and 75 % (v:v) H_2SO_4) was then added to a 50 µL sample of the extract and incubated in boiling water for 7.5 minutes, after which it was cooled down on ice immediately. The absorbance was measured at 625 nm using a spectrophotometer (UV-6300PC, VWR international bvba, Leuven,

Belgium). The total non-structural carbohydrate concentration was calculated using a calibration curve based on solutions containing known amounts of glucose covering the range of the samples' absorption.

Data analysis

All statistical analyses were conducted in R (R Development Core Team 2014) using the *car* and *nlme* packages. For the two oxygen experiments, the mean of data from plants in the same aquarium was taken as one replicate. The adventitious root (AR) number and longest AR produced during complete or partial submergence at different time points were analysed by two-way repeated measures ANOVAs (type III), with oxygen concentration and time as fixed factors and time nested within plant as a random factor. To compare whether the final number of adventitious roots differed between different oxygen concentrations under both partial and complete submergence, one-way ANCOVAs (type III) were conducted with oxygen concentration as a fixed factor and number of primordia as a covariate to compensate for the difference caused by unequal number of adventitious roots. One-way ANOVAs (type III) were conducted on the final total length and total biomass of the ARs, with oxygen as a fixed factor. The effect of oxygen concentrations on final number of ARs was analysed using poisson regressions. P values were adjusted to compensate for multiple comparisons.

For the auxin experiment, one-way ANOVAs (type III) were performed to examine the effects of NAA treatment on the percentage of AR outgrowth and total number of ARs. The total length of ARs was tested using a non-parametric Kruskal-Wallis rank sum test, as the data showed non-homogeneous variances. To meet the assumptions of poisson regression, AR number were rounded up to the nearest integer. To increase the homogeneity of variance, hexose concentration in leaves after flooding and number and length of ARs were *ln* transformed when needed. A relatively small value, i.e., "1", was added to the number of ARs before *ln* transformation to get rid of "0" values.

For the carbohydrate experiment, the number of ARs and longest AR produced at different time points were analysed using three-way repeated measures ANOVAs (type III) with pre-treatment, shade treatment and time as fixed factors, and time nested within plant as a random factor. The total number of ARs were analysed in a two-factor poisson regression with pre-treatment and shade treatment as fixed factors. The final AR length and biomass were analysed in two-way ANOVAs (type III) with the same fixed factors as in the poisson regression. The initial (before flooding) and final

(after flooding) hexose concentrations in the leaf and stem before flooding were analysed in one-way (fixed factor: pre-treatment) and two-way (fixed factors: pre-treatment and shade treatment) ANOVAs (type III), respectively.

Results

Oxygen

In this experiment, plants were either completely or partially submerged with different oxygen concentrations in the floodwater. The effect of the oxygen concentration in the floodwater on adventitious root formation in *S. dulcamara* plants depended on the depth of flooding. Under complete submergence, there was no overall effect of oxygen on the rate of adventitious root development over the seven days of the experiment (Fig. 1, Table 1; $p = 0.530$). However, doubling the oxygen concentration from 21 kPa to 42 kPa (marginally) significantly increased the final adventitious root number, total adventitious root length and dry weight (Fig. 1a & Fig. 2). Under partial flooding, with the major part of the shoot exposed to the atmosphere, plants formed about ten-fold more roots than when they were completely submerged. Reducing the oxygen concentration by flushing nitrogen to the floodwater did not affect adventitious root formation and growth under these conditions (Fig. 3 and 4, table 2). In both oxygen experiments, all plants irrespective of oxygen concentration in water started to form adventitious roots after two days of flooding (Fig. 1 & 3). These experiments suggested that adventitious root formation was not limited by external oxygen concentrations in the floodwater, since increased concentrations did not release the inhibition in outgrowth during complete submergence, and lower concentrations did not inhibit adventitious roots during partial flooding.

Auxin

Local auxin supply to adventitious root primordia under complete submergence significantly increased the percentage of adventitious root outgrowth from 14 % to 69 % (Fig. 5a). In addition to the facilitation of such local adventitious root formation, outgrowth of the root primordia outside the cuvette in which the auxin was supplied, was also stimulated, resulting in a more than 5-fold increase in total number of adventitious roots compared to the control treatment (Fig. 5b). Moreover, auxin supply led to about 20-fold increase in total length of adventitious roots in plants treated with auxin compared with those in the control treatment (Fig. 5c). Consequently, the inhibition of adventitious root outgrowth was released by external auxin supply, suggesting that insufficient

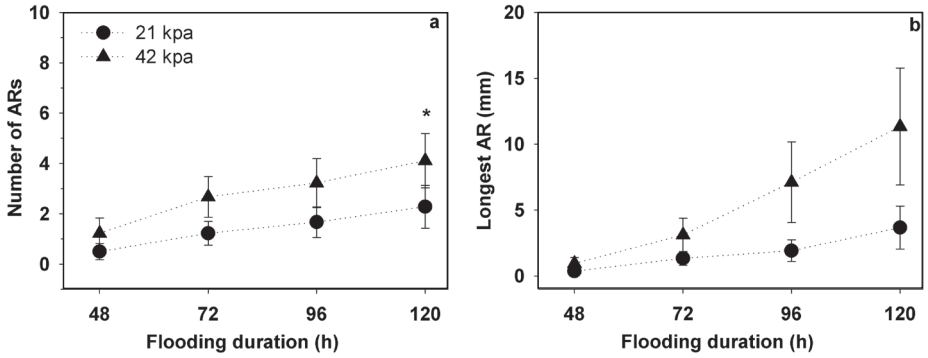

Figure 1. Time course of number of adventitious roots (ARs) (a) and length of the longest adventitious root (b) formed at different oxygen concentrations in the floodwater during 7 d of complete submergence. A significant difference for the final number of adventitious roots between the two oxygen concentrations is indicated by an asterisk (*). P values were adjusted to correct for multiple testing, * $p < 0.025$. Data are means ± (1 SE, n = 9).

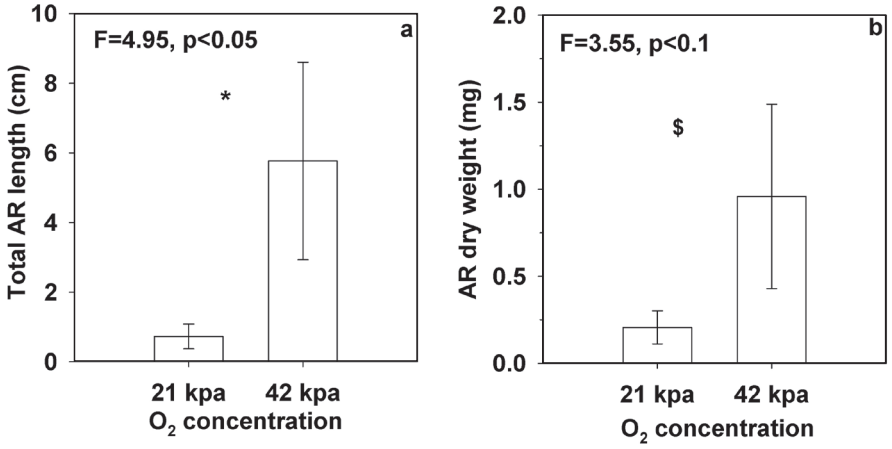

Figure 2. Total length (a) and total dry weight (b) of adventitious roots formed at different oxygen concentrations in the floodwater after 7 d of complete submergence. (Marginally) Significant differences between the two oxygen concentrations are indicated: * $p < 0.05$, $ $p < 0.1$. Data are means ± (1 SE, n = 9).

Table 1 Results of repeated measures ANOVA testing the effects of oxygen concentrations on the number of adventitious roots and the longest adventitious roots along a time series under complete submergence.

	Chi-squares		
	d.f.	**AR number**	**Longest AR**
Oxygen (O)	1	0.395^{ns}	0.537^{ns}
Time (T)	3	3.148^{ns}	**138.262*****
O × T	1	1.054^{ns}	4.075^{ns}

Table 2 Results of repeated measures ANOVA testing the effects of oxygen concentrations on the number of adventitious roots and the longest adventitious roots along a time series under partial flooding.

	Chi-squares		
	d.f.	**AR number**	**Longest AR**
Oxygen (O)	1	0.057^{ns}	0.147^{ns}
Time (T)	3	**359.829*****	**1081.220*****
O × T	1	6.540^{ns}	**23.809*****

Table 3 Results of two-factor poisson regression and two-way ANOVA testing the effects of pre-treatment and shade treatment on the total number, total dry weight and total length of adventitious roots. Values in bold indicate (marginal) significant results.

	Chi-squares / F values					
	d.f.	**Hexose in leaf**	**Hexose in stem**	**Total AR number**	**AR dry weight**	**Total AR length**
Pre-treatment (Pre)	1	0.622^{ns}	0.104^{ns}	2.441^{ns}	1.157^{ns}	**6.015***
Shade (S)	1	**180.912*****	**30.067*****	**22.046*****	**23.788*****	**9.718****
Pre × S	1	1.329^{ns}	1.003^{ns}	**12.246*****	**3.811**$^{\$}$	**7.268***

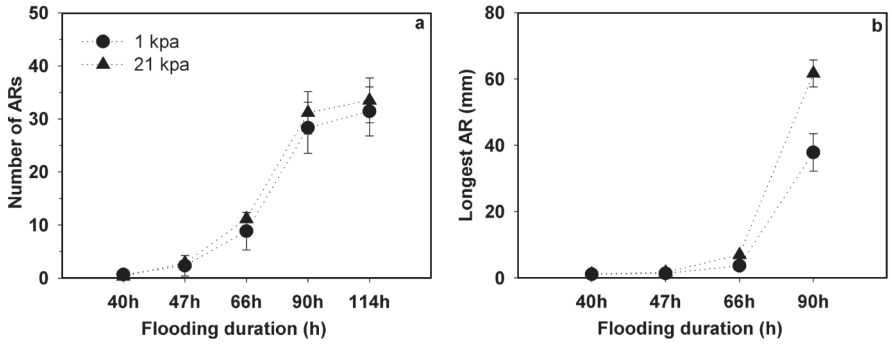

Figure 3. Time course of number of adventitious roots (a) and length of the longest adventitious root (b) formed at two different oxygen concentrations in the floodwater during 7 d of partial flooding. Data are means ± (1 SE, n = 12).

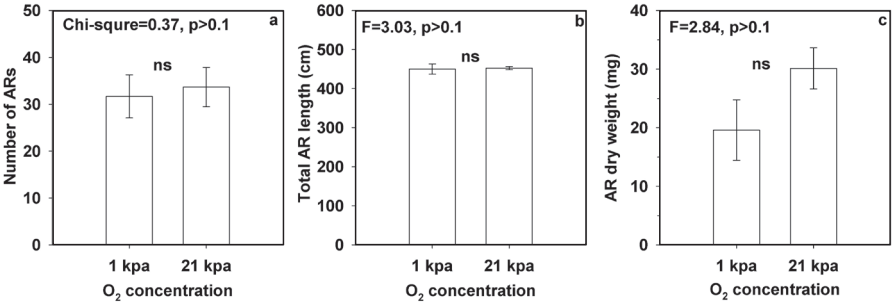

Figure 4. Total number (a), total length (b) and total dry weight (c) of adventitious roots formed at different oxygen concentrations in the floodwater after 7 d of partial flooding. Results and significance of one-way ANCOVA (a) and one-way ANOVAs (b & c) are provided. Data are means ± (1 SE, n = 12).

auxin delivery at the root primordia may be a key limitation for adventitious root formation during complete submergence.

Carbohydrate

Carbohydrate depletion prior to flooding significantly reduced the hexose concentration in plant leaves but not in the stem (Fig. 6a). At the end of the flooding period, the pre-treatments did not affect the final hexose concentration in either leaves or stems, but shading during flooding significantly decreased the hexose concentration in both leaves and stems (Fig. 6b, table 3). This

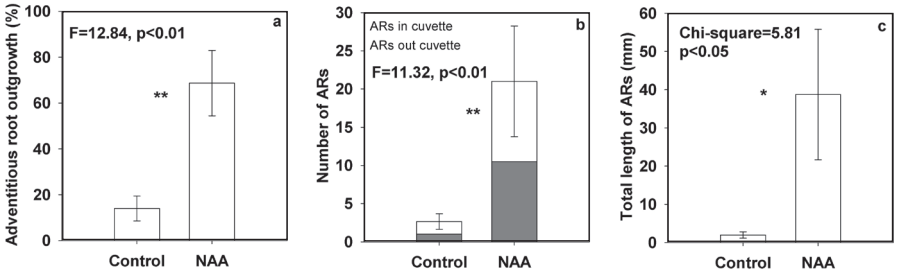

Figure 5. Percentage of adventitious root outgrowth (a), number (b) and total length (c) of the adventitious roots formed in control and NAA treatments after 10 d of complete submergence. Results and significance of one-way ANOVAs (a & b) and Kruskal-Wallis rank sum test (c) are provided. Data are means ± (1 SE, n = 6).

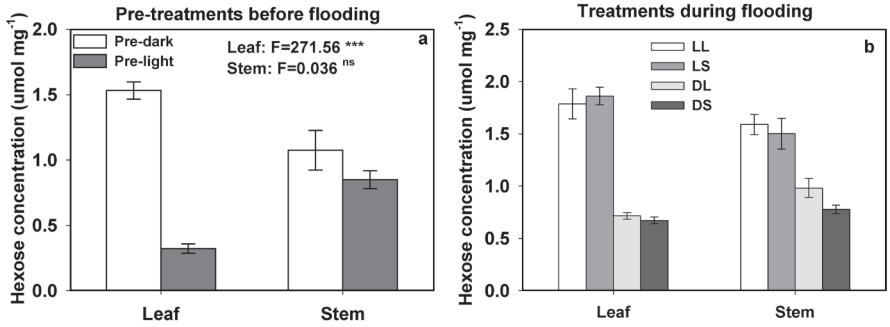

Figure 6. The total hexose concentrations in leaf and stem tissues after two days of pre-treatments prior to flooding (a) and after being partially flooded under shaded (LS & DS) and non-shaded conditions (LL & DL) for 10 d (b). Results and significance of one-way ANOVA testing effects of pre-treatments on the hexose concentration prior to flooding are provided (a): *** $p < 0.001$, ns $p > 0.1$. Data are means ± (1 SE, n = 8 to 10).

suggests that the most recent light conditions mainly contribute to the present carbohydrate status. Pre-depletion of carbohydrates did not affect the number and dry weight of adventitious roots and the length of the longest adventitious root produced during the seven days of flooding (Fig. 7, table 4), but significantly increased the total length of the adventitious roots (Fig. 8, table 3). These results suggest that adventitious root development may be dependent on the carbon content in plant stem. Shading during flooding had significant effects on all the four variables (Fig. 7a, table 4); however, the negative or positive effects of shading depended on carbohydrate depletion in the pre-treatments

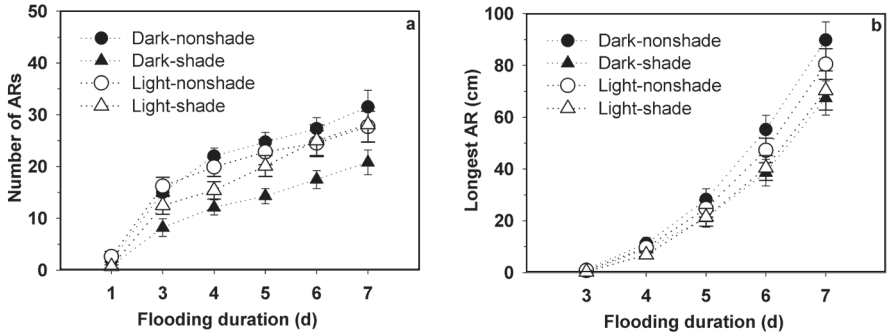

Figure 7. Time course of number of adventitious roots (a) and length of the longest adventitious root (b) formed in four different carbohydrate manipulation treatments (dark-nonshade, dark-shade, light-nonshade and light-shade) during 7 d of partial flooding. Data are means ± (1 SE, n = 10).

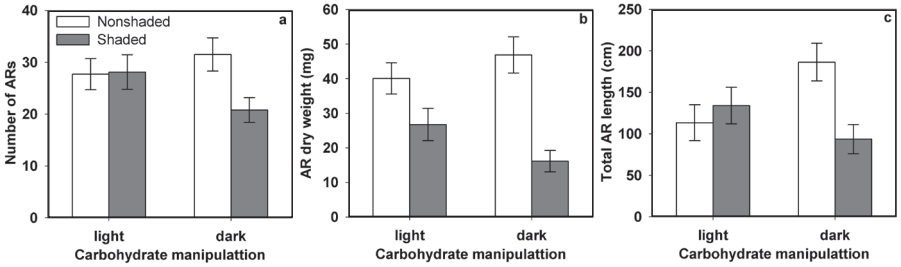

Figure 8. Total number (a), total length (b) and total dry weight (c) of adventitious roots formed in four different carbohydrate manipulation treatments (dark-nonshade, dark-shade, light-nonshade and light-shade) during partial flooding. Data are means ± (1 SE, n = 10).

Table 4 Results of repeated measures ANOVA testing the effects of pre-treatment, shade treatment and time on the number of adventitious roots and the longest adventitious roots during 10 d of partial flooding.

		Chi-squares	
	d.f.	**AR number**	**Longest AR**
Pre-treatment (Pre)	1	0.5976^{ns}	0.007^{ns}
Shade (S)	1	**4.136***	0.020^{ns}
Time (T)	5	**445.271*****	**639.822*****
Pre × S	1	0.163^{ns}	0.002^{ns}
Pre × T	5	3.988^{ns}	3.776^{ns}
S × T	5	2.206^{ns}	**21.840*****
Pre × S × T	5	5.001^{ns}	4.022^{ns}

(Table 3). Shading significantly decreased the total number and total length of adventitious roots of plants whose carbohydrate were depleted before flooding, but it did not affect these two variables in plants that were previously grown under ambient light conditions (Fig. 8a, table 3). The total dry weight of adventitious roots was significantly reduced by shading and this effect was stronger if the carbohydrates of plants were previously depleted (Fig. 8b, table 3). The significant interactions between the light conditions experienced prior to flooding and light conditions during flooding on adventitious root formation ($p = 0.005$, $p = 0.011$ and $p = 0.059$ for number, dry weight and total length of adventitious root, respectively) indicates that although the light conditions prior to flooding might not directly influence the initiation of adventitious roots, they did influence the growth of these roots after initiation.

Discussion

Flooding is a compound stress that imposes a variety of environmental limitations to plants, such as oxygen shortage and shading (especially in turbid water), commonly leading to endogenous limitations such as energy deficiency that arrest plant growth and development (Bailey-Serres & Voesenek 2008). Plants occurring in flood-prone areas have evolved a suite of adaptive responses that alleviate the negative impact of flooding (Colmer & Voesenek 2009; Bailey-Serres, Lee & Brinton 2012). However, when plants are completely submerged, the environmental and endogenous limitations associated with flooding are predicted to become more severe (Voesenek *et al.* 2006) and thereby inhibit the optimal expression of adaptive traits (Zhang *et al.* 2015). While the factors inducing adaptive changes to flooding are typically well known, the mechanisms constraining the expression of plastic responses are much less well understood. In this study, we used *Solanum dulcamara* to identify the limiting factor(s) for adaptive adventitious root formation during complete submergence. Adventitious root formation is a common adaptation observed in a wide range of species when flooded (Colmer & Voesenek 2009). Given the clear benefits associated with adventitious roots in flooded plants (Chapter 3), adventitious root formation is thought to be of adaptive value for species that are more frequently exposed to flooding. However, a previous study showed that the formation of adventitious roots in *S. dulcamara* is a trait that is only expressed during partial flooding but not under complete submergence (Zhang *et al.* 2015), which may limit plant establishment in areas subjected to recurrent flooding. We manipulated the dissolved oxygen concentration in the floodwater and plant carbohydrate availability, which are potentially essential

resources for adventitious root growth, and externally applied auxin, a positive regulator of adventitious root formation, the production and transport of which might be limited under severe oxygen deficiency, to gain insight into the mechanisms that impede adventitious root production under complete submergence. Here, we will discuss the effect of each of these three factors separately.

Shoot atmospheric contact is essential for adventitious root formation in *S. dulcamara*

Adventitious root growth is sensitive to oxygen deficiency because it requires energy from respiration for cell division and elongation (Visser *et al.* 1997; Sauter 2013; Dawood *et al.* 2014). However, it is not well understood whether adventitious root growth mostly relies on internally or externally derived oxygen. In the present study, increasing the external oxygen concentrations in the floodwater significantly promoted adventitious root formation during complete submergence, whereas in a previous study adventitious root formation was inhibited in plants completely flooded in stagnant water (Zhang *et al.* 2015). This indicates that some influx of external oxygen would be beneficial for adventitious root formation in completely submerged *S. dulcamara*. Similar results have been shown in plant cuttings. Lowering the concentrations of dissolved oxygen in hydroponics resulted in lower rooting percentage, fewer and shorter roots and longer time for root emergence in cuttings of a woody and a herbaceous species (Soffer & Burger 1988). Nevertheless, the effect size of external oxygen increment on adventitious root formation in the present study is quite small in comparison with that of shoot exposure to air in partial flooding. On the other hand, artificially depleting external oxygen concentrations in the water in partially flooded plants did not negatively affect adventitious root development. Taken together, these results provide evidence that shoot atmospheric contact is the more efficient oxygen source for adventitious root development, oxygen from the water, however, potentially due to diffusive barriers, may not be able to reach the primordia, thus less efficient for adventitious root growth. However, further studies explicitly measuring oxygen status in the adventitious root primordia while changing the external oxygen concentrations in floodwater are needed in order to provide more conclusive answers.

Similar results were found for root growth in rice, where root extension completely relies on internal oxygen transport within the plant from the atmosphere (Armstrong and Webb, 1985). In contrast with *S. dulcamara* and rice, low oxygen concentrations (< 5 %) were shown to impede lateral root

elongation in *Rumex thyrsiflorus* and *R. palustris* and nodal root elongation in wheat (Visser *et al.* 1997; Wiengweera & Greenway 2004). Lowering the surrounding oxygen of the shoot had additive effects on the root elongation rates in all three species (Visser *et al.* 1997; Wiengweera & Greenway 2004). This difference in oxygen effects on root development among different species may relate to the oxygen permeability of the epidermis as well as on the extent to which the flooded plant tissues rely on each of the two oxygen sources, internal oxygen transport from the atmosphere and dissolved oxygen from the surrounding water.

Auxin limitation might suppress adventitious root formation during complete submergence

Auxin is a central regulator for lateral and adventitious root development (Casimiro *et al.* 2001; Bhalerao *et al.* 2002; Grieneisen *et al.* 2007; Steffens & Rasmussen 2016). It was already a few decades ago shown by Wilkins and Martin (1967) that the basipetal polar transport of auxin can be inhibited by low oxygen conditions, therefore, the auxin concentration in the adventitious root primordia on the basal stem may be expected to be lower during complete submergence than during partial flooding. As the metabolism is inhibited under anaerobic conditions, auxin synthesis, which is an energy-demanding processes (Jones 1998; Vieten *et al.* 2007), may also be slowed down during complete submergence. Our result that exogenous application of auxin greatly improved the adventitious root formation suggests that auxin limitation in the primordia may be one of the key factors limiting the adventitious root formation during complete submergence. Unexpectedly, without direct contact, auxin application also stimulated the outgrowth of the primordia above and underneath the cuvette, likely indicating internal auxin movement after the auxin entered the stem. This contradicts the hypothesis that oxygen shortage limited auxin transport, but possibly at sufficiently high concentrations and small distances, some diffusion from the stem part that was exposed to the auxin solution is still possible.

Newly synthesized but not stored carbohydrates stimulate adventitious root development during flooding

Complete submergence of the entire shoot further decreases light availability, leading to a substantial decline in photosynthesis (Mommer & Visser 2005; Voesenek *et al.* 2006). As a result, carbohydrate production is expected to be strongly limited during complete submergence. Non-structural carbohydrates (NSC) have been shown to be essential for plants that employ a quiescence

strategy to survive deep flooding (Bailey-Serres & Voesenek 2008). They may also influence the plant's plasticity in response to flooding; e.g., shoot elongation, a common plastic response to flooding, has been shown to strongly correlate with the carbohydrate status of the plant (Groeneveld & Voesenek 2003; Das, Sarkar & Ismail 2005; Chen *et al.* 2011; Huber *et al.* 2012). Compared to shoot elongation that mainly results from cell elongation, adventitious root formation, which consists of both cell elongation and division, is an even more energy-consuming process. Therefore, adventitious root formation is expected to be more reliant on carbohydrate availability. In our study, adventitious root formation was closely correlated with the concentration of NSC in the shoot of the plant after flooding. However, to our surprise, the initial NSC concentration in leaves, before flooding, seemed to have only little effect on the adventitious roots except on the final length of these roots. Compared to the carbohydrates in leaves, carbohydrates in the local stem did not change, which may suggest that carbohydrates in the local stem might be directly available for adventitious root growth, and therefore be expected to be more important than the leaf carbohydrate content. Studies on cuttings showed that increasing local carbohydrate concentration in the rooting zone of the stem by direct sugar and auxin applicationsignificantly enhanced adventitious root formation (Altman & Wareing 1975). However, unlike the stronger dependence of adventitious root formation on the carbohydrate content in stems than in leaves, carbohydrates in different organs of *R. palustris* plants showed similar positive effects on shoot elongation (Huber *et al.* 2012). This difference may suggest that the processes contributing the initiation of adventitious root formation are more localized than the processes triggering shoot elongation in a rosette plant. Further studies investigating the role of direct carbohydrate source for adventitious root formation are needed to better understand the effects of carbon on this process. Additionally, the conversion from starch to glucose may be difficult during complete submergence, as the activity of enzymes that are responsible for starch degradation may be inhibited under low oxygen conditions (Guglielminetti *et al.* 1995), thereby limiting access to stored carbohydrates (mainly starch). In contrast, the major form of the newly formed carbohydrates through photosynthesis is sucrose, which can directly be used in respiration. Similar results have been found for shoot elongation in rice, where the initial carbohydrates had little impact on the elongation ability, whereas carbohydrates present in plants immediately after flooding showed a strong positive correlation with this trait (Das, Sarkar & Ismail 2005). Therefore, identifying the direct origin of different resources is important for understanding the role that resource plays in an adaptive plastic response during flooding.

Conclusions

Due to the complex nature of complete submergence, unravelling the key factor(s) limiting the expression of adaptive traits in plants proves to be challenging. As environmental limitations associated with complete submergence interact and cause different internal constraints, the final outcome of flooding acclimations is determined by multiple factors. For adventitious root formation, we found that limitation in oxygen, carbohydrate and auxin content all played a role in the lack of adventitious root formation of *Solanum dulcamara* during complete submergence. This suggests that as plants are integrated organisms, not a single factor is responsible for a given response. These three factors may interact with each other, synergistically leading to lack of adventitious root formation. For instance, oxygen and carbohydrate limitation may hamper plant growth, leading to lower auxin production thereby impeding adventitious root formation. Awareness of complicated between-factor interactions asks for more studies manipulating the various factors together to consider these interactions when studying the complex nature of plant responses. Under natural conditions, the limited expression of adaptive adventitious root formation in response to complete submergence may limit the distribution of this species to only occupying habitats that are subjected to shallow flooding, such as shores of dune slacks, mountain streams and shallow inland lakes.

Chapter 6

Habitat of origin does not influence rooting patterns and response to drought in *Solanum dulcamara*

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Abstract

Drought is a severe environmental stress in various ecosystems. Improved water acquisition through an optimised root architecture has been reported to benefit plant drought resistance. Such root-architectural adaptive traits are either expressed constitutively during root development or induced by local drought events. However, plant populations from habitats contrasting in drought occurrence may differ in these constitutive and plastic root traits, as wetter habitats may select for different root architecture. To test this hypothesis, we first screened the early constitutive rooting patterns with nine pairs of *Solanum dulcamara* populations originating from contrasting (flooding- and drought-prone) habitats in non-drought conditions. Three out of the nine pairs showed slight differences in constitutive root traits, although most of these were not statistically significant. To investigate whether these three pairs of populations differed in root plasticity and also whether the differences in early constitutive root traits could be translated into differences in drought resistance in later developmental stages, we subjected the three pairs to control and drought treatments in long tubes in a greenhouse experiment for seven weeks. The results showed little difference in root traits such as rooting depth and root biomass distribution along soil depth neither under control nor under drought conditions. In conclusion, we showed that habitat of origin did not lead to divergent selection in neither constitutive nor plastic root growth in *S. dulcamara*. These results provide additional evidence to previous findings that local adaptation to flooding and drought did not occur in this species.

Introduction

Water shortage is a severe environmental constraint to plant productivity (Bray 1997; Comas *et al.* 2013), which causes a cascade of physiological consequences for the entire plant (Farooq *et al.* 2012). The risk of drought stress is predicted to increase due to climate change (IPCC 2013). Sustaining plant growth under prolonged drought stress is essential for various ecosystems, including agricultural lands, grasslands as well as forests (Passioura 2007). Under progressive soil drying, both water and nutrient availability gradually become limited (Lynch 2013). As a result, an extensive root system that increases belowground resource exploration is predicted to increase plant survival and performance (Lynch 1995). In addition, deep rooting provides the plant with better access to sub-soil ground water, allowing improved performance during topsoil drought (Wasson *et al.* 2012; Maeght, Rewald & Pierret 2013).

The plant traits that improve drought tolerance may be quite diverse (Farooq *et al.* 2012). For instance, increased root to shoot ratio is commonly observed in drought resistant plant species (Jaleel *et al.* 2009; Poorter *et al.* 2012; Pierik & Testerink 2014). Such rapid biomass allocation to the root system upon drought events is essential for continuation of plant growth under drought (Poorter *et al.* 2012) and an increased allocation of root biomass towards deeper growing roots may be an even more crucial trait (Lynch 1995). Substantial evidence shows that such deep rooting can improve water uptake during drought stress and is therefore correlated with high drought resistance (Kell 2011; Wasson *et al.* 2012; Lynch 2013). For instance, introducing the deep rooting quantitative trait loci DEEPER ROOTING 1 from a deep-rooting rice cultivar to a shallow-rooting cultivar significantly increased the root length of the latter, and thereby substantially improved its drought resistance (Uga *et al.* 2013). Seedling mortality of five Mediterranean woody species strongly correlated with rooting depth (Padilla & Pugnaire 2007). Studies with crop species, such as wheat, maize and bean, suggested that genotypes with deeper root systems had higher yield potentials than those with shallow root systems under drought conditions (Ho *et al.* 2005; Manschadi, Christopher & Hammer 2006; Fenta *et al.* 2014; Nyakudya & Stroosnijder 2014).

In addition to deep rooting, also other root traits may contribute to enhanced soil exploration for water, thus cannot be excluded (Lynch 2011). As water may be heterogeneously distributed in the soil, particularly when gradually depleted in the local rhizosphere, characteristics of the root system that increase the range of root foraging both vertically and horizontally may significantly improve water uptake (Asch *et al.* 2005; Hund, Ruta & Liedgens

2009; Lynch 2011). E.g., increased root volume and root length, steep root angles as well as horizontal root expansion enable plant roots to explore more volume of soil and are likely to stimulate the opportunity of water capture (Lynch 1995; Lynch 2013). Plant species and even different genotypes within a species differ substantially in their root architecture. Such intrinsic difference in the adaptive rooting traits may translate into different interspecific and/or intraspecific drought resistance.

The root system of a plant is rather plastic (Lynch 1995). High root architectural plasticity in e.g. root dry weight, root length density and % lateral roots was found in rice plants, which was closely related with yield (Sandhu *et al.* 2016). Screening on rooting depth of 42 vascular species revealed various levels of plasticity in rooting depth in 12 species in response to drought, and differences in the degree of plasticity seemed to be linked to the selective pressures in the habitat of the species (Reader *et al.* 1993). These results suggest that plasticity in drought related rooting traits is under selection (O'Toole & Bland 1987; Malamy 2005). Deep rooting has been argued to be beneficial when the topsoil dries out but soil moisture is maintained in deeper soil layers (Jaleel *et al.* 2009). On the contrary, under moist conditions, nutrient acquisition which is associated with topsoil foraging (Ho *et al.* 2005) may require a shallow root distribution. Especially the availability of phosphorous which is immobile is greatest in the topsoil and decreases with soil depth (Jaleel *et al.* 2009), thus requiring a contrasting root system phenotype (shallow rooting) than the ideal drought phenotype (deep rooting). In addition, when excess water is present in the soil, oxygen concentrations rapidly decline due to the slow gas exchange between soil and water (Colmer & Voesenek 2009), and, as a result, shallow roots close to the soil surface are more likely to survive this oxygen deficiency stress (Stefan & Strand 1996; Chen, Qualls & Miller 2002). Therefore, plants occupying habitats of contrasting soil moisture may be subjected to selection pressures favouring opposing adaptive root traits and hence different drought tolerance. Species inhabiting habitats over the entire soil moisture gradient, while being scarce, can be expected to have formed distinctly different ecotypes, each with a different root system architecture. Although variation among genotypes in supposed drought tolerance related root traits has been reported (Ho *et al.* 2005; Manschadi, Christopher & Hammer 2006; Fenta *et al.* 2014), it is yet unclear whether such differences can indeed be linked to the local soil moisture conditions (wet or dry).

Solanum dulcamara is a species that inhabits contrasting, i.e., drought- and flooding-prone, habitats in the Netherlands, namely the top of dry sand dunes and the wet shores of dune slacks. On the dunes where the soil water content

in the top soil (< 0.5 m deep) can be as low as 2-3 % (v:v) already in spring, *S. dulcamara* has been observed to root to over 1.5 m deep (personal observation E.J.W. Visser), whereas on the shores of dune slacks in shallow water this species produces a large quantity of superficially growing adventitious roots. The consequences for growth in wet and dry conditions were explored earlier, in Chapter 2, where we also found significant genetic differentiation between populations from the two contrasting habitat types. In Chapter 2, it became apparent that variation in adventitious root formation upon flooding did not differ between habitats, and that root biomass allocation in response to drought did not differ between dry and wet habitats either. However, the latter was tested in relatively small and shallow pots that quickly dried out over their entire depth, hence plants could not escape from drought by producing deeper roots.

Here we hypothesize that *S. dulcamara* plants originating from the two contrasting habitat types differ significantly in their rooting patterns and the way they may change this pattern upon drought and flooding stress, each rooting pattern being favoured in the local habitat. Experiments were set-up that tested explicitly if different rooting architecture exists among populations of *S. dulcamara* from different habitats. We expect constitutive differences in rooting architecture to be already manifested in early developmental stages, as these stages may be essential for plants to cope with increasingly dry conditions in the course of the growing period. Typically constitutive variation in root characteristics is often screened in young seedlings in non-stressed conditions (Gregory *et al.* 2009), e.g., in crop research (e.g., O'Brien 1979; Sanguineti *et al.* 2007; Manschadi *et al.* 2008; Watt *et al.* 2013). Alternatively, the adaptive root traits might be a result of rapid plastic responses that are induced under stress conditions (e.g., Wang *et al.* 2009), and therefore can only be found in plants subjected to drought stress. To test the hypothesis that plants of dry habitats have either constitutively deeper or larger root systems, or respond faster or stronger with deep rooting upon drought stress, or a combination of both, we used the same nine pairs of populations from dry and wet habitats as in Chapter 2. We first investigated the constitutive rooting patterns by screening the early development of root systems of seedlings on germination paper. However, as environment may have strong impacts on the plasticity in root traits, whether the constitutive differences in these traits remain under drought conditions is not clear. Therefore, we subsequently selected three population pairs with the largest contrast in constitutive rooting patterns to investigate whether differences in early rooting patterns also translate into difference in rooting depths in plants under drought stress. These plants were subjected to drought

in a greenhouse experiment with the option of developing a deep root system and reach moist soil at greater depth.

Materials and methods

Seed germination and plant growth

Root screening experiment

Seeds of *Solanum dulcamara* originating from nine pairs of populations collected in contrasting habitats were used in this experiment (details see chapter 2). A hundred seeds from each of the 18 populations were surface-sterilized in 15 % (w:v) tri-sodium phosphate solution for 20 min and subsequently rinsed with abundant tap water before being sown on wet filter paper in petri dishes. The seeds were first stored at 4 ˚C for eight weeks to break dormancy, and then placed at 21 ˚C in the light for germination.

Drought experiment

Three out of the nine pairs of populations were selected according to differences in their rooting patterns on germination paper, i.e. the locations Schiermonnikoog (S), Texel (T), and Zandvoort (Z). Eighty seeds of each population were treated in the same manner as in the 'root screening experiment' before being sown on wet filter paper in petri dishes. The seeds were first stored at 4 ˚C for one week to break dormancy, and then moved to 21 ˚C in the light for germination. After ten days, 25 homogeneous seedlings from each population were transplanted into individual round deep pots (volume 250 ml, depth 15 cm) filled with sieved commercial compost (Horticoop substrate, Lentse potgrond and Slingerland potgrond, Cuijk, the Netherlands).

Experimental design

Root screening experiment

Four days after germination, before the emergence of the cotyledons, 24 seedlings with homogeneous root length from each population were distributed over 108 aluminium plates (length × width × depth, 32 × 26 × 2 cm) lined with wet germination paper. The germination paper (Anchor Steel Blue Seed Germination Blotter, Anchor Papers Co, Saint Paul, Minnesota, USA) was first soaked in a nutrient solution (2 mM $Ca[NO_3]_2 \cdot 4\ H_2O$, 1.25 mM K_2SO_4, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, and micronutrients NaCl 50 µM, H_3BO_3 25 µM, $MnSO_4$ 2 µM, $ZnSO_4$ 2 µM, $CuSO_4$ 0.5 µM, H_2MoO_4 0.5 µM, Fe(3+)-Na-EDTA 90 µM) and then carefully placed on the plate. This paper provided water and nutrients to the seedlings placed on it. For each plate, two pairs of seedlings

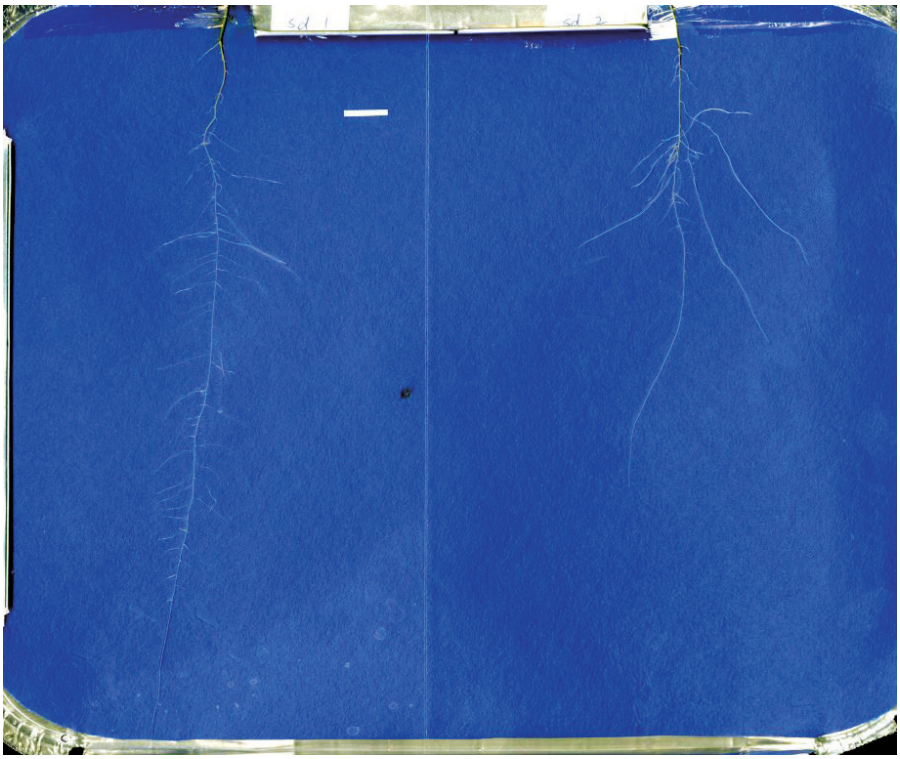

Figure 1. Contrasting constitutively determined root architecture of two-week-old seedlings of *S. dulcamara* after two weeks of growth: root system with long primary root and short lateral roots (left) and root system with short primary root and long lateral roots (right).

were placed at about 8 cm from each side of the plate. The distance between the seeds and the top of the plate was 2-3 cm, leaving the rooting depth, to the bottom of the plate, to about 23 cm. After placing the seedlings on the blue germination paper, two wet filter paper strips (5 cm long, 3 cm wide) were placed horizontally across the base of the roots to attach the seedlings and their root system firmly to the germination paper. Each plate was covered by another aluminium plate to create dark conditions for the roots and to keep a relatively high humidity in the rooting zone. Two holes above the seeds on the top end of the plate were made to enable shoots to grow above the plate. All 108 plates were assigned to nine plastic trays (length x width x depth, 39.5 x 29.5 x 20 cm) with three pairs of populations in three trays, each containing two plates from the same population. Nutrient solution was added to each tray to a height of 5 cm to provide water and nutrients. After four days, the filter

paper strips were removed and the four seeds were thinned to two (one on each half of the plate; Fig. 1). The experiment lasted for four weeks, during which the temperature was kept at 21 ˚C. The nutrient solution was kept at 5 cm in the trays throughout the experiment.

Drought experiment

This experiment consisted of two irrigation treatments (control and drought) and three population pairs (populations from both wet (W) and dry (D) habitats in each of the three locations: Schiermonnikoog (S), Texel (T) and Zandvoort (Z)). These three pairs were chosen for their visually apparent difference in rooting pattern between habitats. As we were not specifically interested in effect of location, this factor functions as replicates in order to obtain general patterns. Each combination of treatment and population contained eight replicates, resulting in 96 plants in total. At four weeks old, 16 equal-sized seedlings from each population were selected and transplanted together with their substrate into individual PVC tubes (diameter 7 cm, height 130 cm) filled with a mixture of pure sand and 4 g L^{-1} slow-release fertilizer (Osmocote® Exact Standard, 16-9-12+2 MgO+TE, release time 3-4 months, Everris International B.V., Geldermalsen, the Netherlands). After transplanting, the substrate was covered by a 2-3 cm thick layer of sand in each tube. The initial water content in the tube was 4.3 ± 0.08 % (v:v, n=8). Forty-eight tubes containing 48 individual plants for each irrigation treatment were assigned to four containers (length x width x height, 98 x 98 x 28 cm), each containing two plants from each of the six populations. The tubes were randomized and fixed to a plastic cubic block in the centre of each container. The distance between adjacent tubes was kept at a minimum of 10 cm to avoid shading from the neighbouring plant in later growth stages. After being transplanted into the tubes, each plant received 50 ml nutrient solution (2 g L^{-1} Kristalon in rain water, Yara International ASA, Vlaardingen, the Netherlands) within the first week of the experiment to prevent nutrient deficiency at the onset of the experiment. Each plant in the drought treatment received 150 ml rain water within the first ten days. From then on, each plant in the drought treatment received 100 ml rain water every time when more than half of the plants showed mild wilting (only lower leaves losing turgor) or when at least one plant showed severe wilting (≥ 2/3 leaves losing turgor). Plants in the control treatment were watered every day with ample rain water. Apart from watering from the top of the tubes, each tube in both treatments received water from the bottom by keeping the water level in the container at approximately 15 cm. This raised the water table in the sand inside each tube through capillary rise to about 30-40 cm from the bottom (i.e., at ca. 90 cm below the soil surface), thus

mimicking the presence of deep water in the dry dune habitat. The experiment lasted for seven weeks and plants were harvested when more than half of the plants had reached the bottom water table.

Measurements

Root screening experiment

Root scans (Fig. 1) were taken at the end of the first, second and third week of the experiment, and when the roots reached the bottom of the plate, using an A3-sized flatbed scanner (Expression 11000XL, Epson, Japan). Since the majority of primary roots reached the bottom of the plate at the end of the second week, these scans were used to calculate rooting depth, rooting width, rooting area, total root length, the longest lateral root and number of lateral roots in WinRHIZO (Regent Instruments Inc., Québec, Canada). Rooting depth was defined as the length of the main root, rooting width as the distance between the two most laterally exterior root tips or root parts, and rooting area as the area covered by the polygonal drawn between all most exterior root tips or parts. Scans from three (SW *vs.* SD, TW *vs.* TD & ZW *vs.* ZD) out of nine population pairs were analysed for their visually apparent difference in rooting pattern between habitats.

Drought experiment

One fully expanded leaf was collected from each plant one day before the final harvest, and then scanned using a A3-sized flatbed scanner (Expression 11000XL, Epson, Japan) to determine its surface area in WinRHIZO (parameter "projected area"), which was then used to calculate a specific leaf area (SLA). During harvest, shoots were first cut off, after which the entire root column with all the substrate was carefully pushed out of the tube and laid on a plastic sheet on the ground. The length of the deepest root in the substrate was measured with a ruler. Subsequently, the root column was divided into five fractions measured from the shoot-root conjunction: the top 10 cm, 10-25 cm, 25-40 cm, 40-55 cm and the remaining roots. The five fractions of roots were washed free of substrate, after which each fraction was divided into tap roots and fine roots. A subsample of fine roots from the third fraction (25-40 cm deep) was taken for determination of specific root length (SRL). These roots were soaked in 0.035 % Neutral Red in 70% (v:v) ethanol to preserve and stain the roots, and were subsequently stored at 4 ˚C. The length, surface area, volume and diameter of these roots were determined using WinRHIZO after being scanned. All plant materials were dried for 72 h at 70 ˚C and weighed.

Data analysis

Calculations

To compensate for the difference in sample size of fine roots used for scanning, root surface area (RSA) and root volume were divided by the dry weight of the root sample in the drought experiment. Proportional root length was presented in percentage which was calculated by dividing the root length in each diameter class by the total root length of all diameter classes.

Analyses

All the analyses were performed in R (R Development Core Team 2014), using the *car* and *lme4* packages, type III sum of squares were used in all the analyses. For the root screening experiment, we selected three pairs of contrasting populations originating from Schiermonnikoog (SW *vs.* SD), Texel (TW *vs.* TD) and Zandvoort (ZW *vs.* ZD) to conduct the following analyses, as they appeared to have the largest visually discernible variation in lateral root length between habitats out of the nine habitat pairs. The effects of habitat and location on rooting depth and total root length were tested by means of two-way ANCOVAs (type III), with initial root length as a covariate. The main effects of habitat and location and their interaction on the longest lateral root, number of lateral roots, rooting width and rooting area were tested by two-way ANOVAs (type III). Student's *t* tests were conducted in order to compare the difference between dry and wet habitats within each location. P values were adjusted using the Bonferroni correction to correct for multiple comparisons. Total root length was *ln* transformed prior to the analysis to reduce heteroscedasticity.

For the drought experiment, we were mainly interested in the general patterns from the three locations, rather in the specific responses in each location, and location was treated as a random factor for all analyses. Rooting depth was analysed by means of a split-plot ANOVA (type III) model, with treatment and habitat as fixed factors, habitat nested within location as a random factor. SRL, root surface area (RSA), root volume, average root diameter, SLA, total biomass and root to shoot ratio were analysed in split-plot ANOVAs (type III), with treatment and habitat as fixed factors and habitat nested within location as a random factor. As the main effect of habitat on total biomass and the interaction between treatment and habitat on the average root diameter were significant, student's *t* tests were conducted comparing the difference between dry and wet habitat within each treatment. To test whether treatment and habitat caused differences in the partitioning of roots to different diameters, the percentage of root length calculated from three

Table 1 Results of a two-way ANCOVA testing the effects of habitat and location on the rooting depth and total root length of *S. dulcamara* plants from the screening experiment. Initial root length (RL) was included as a covariate in the model. Results of a two-way ANOVA testing the effects of habitat and location on the rooting width, rooting area, longest lateral root and number of lateral roots of these plants were also shown in the table. Significant and marginally significant F-values were in bold; $^{***}p \leq 0.001$, $^{**}0.001 < p \leq 0.01$, $^{*}0.01 < p \leq 0.05$, $^{\$}0.05 < p \leq 0.1$, $^{ns}p > 0.1$.

		F-values					
	d.f.	**Rooting depth**	**Rooting width**	**Rooting area**	**Total root length**	**Longest lateral root**	**Lateral root number**
Habitat (H)	1	0.254^{ns}	0.882^{ns}	0.507^{ns}	**4.762***	**3.518**$^{\$}$	0.297^{ns}
Location (L)	2	**4.844***	2.200^{ns}	**6.183****	**10.185*****	0.123^{ns}	**3.080**$^{\$}$
H × L	2	**9.223*****	**3.562***	**12.048*****	**12.177*****	0.224^{ns}	0.332^{ns}
Initial RL	1	0.248^{ns}	-----	------	0.141^{ns}	-----	------

root diameter classes (0.0-0.2 mm, 0.2-0.6 mm and >0.6 mm) was analysed by means of a split-plot ANOVA (type III), with treatment, habitat and diameter class as fixed factors and habitat nested within location as a random factor. Tap root biomass, fine root biomass and total root biomass were analysed by split-plot ANOVAs, with treatment, habitat and fraction as fixed factors, and habitat nested within location as a random factor. The percentage of root length per diameter class was also analysed by a split-plot ANOVA, with treatment, habitat and diameter class as fixed factors, and habitat nested within location as a random factor. As there was a significant interaction between treatment and diameter class, student's *t* tests were then conducted within each diameter class to compare the difference between control and drought treatments. P values were adjusted using the Bonferroni correction to take multiple comparisons into account. To reduce heteroscedasticity, SRL and fine root biomass were transformed by raising the data to the power of 4 and 7, respectively, SLA was *ln* transformed, and taproot biomass and total root biomass were square-root transformed. As some plants in the drought treatment did not root into the fifth fraction (> 55 cm), zero was given to that fraction in such cases. Before the square root transformation, the smallest value from taproot biomass (0.0027 g) and that from total root biomass (0.0043 g) were added to the two variables for each plant, respectively, to remove zeros in the data. The percentage of root length was transformed by conducting a *ln* transformation on "A/(1-A)", where A represents the percentage data.

Results

Habitat effects on root traits in the screening experiment

Habitat had no significant effect on most of the root traits of interest except for total root length and longest lateral root (Table 1). Although plants from both habitats produced a similar number of lateral roots, plants from wet habitats tended to produce longer lateral roots than those from dry habitats (Fig. 2e, Table 1) which resulted in a higher total root length in plants originating from the wet habitats compared to those from dry habitats (Fig. 2d, Table 1). No overall habitat effect appeared on rooting depth, rooting width and rooting area across all the three locations (Fig. 2a-c, Table 1). The effect of habitat varied among locations, for example in the case of Zandvoort, plants originating from the dry habitat rooted deeper than those from wet habitat, but this effect was not found in plants from the other two locations. Similarly, for Texel, but not for the other two locations, wet habitat plants were characterized by a significantly larger rooting width and rooting area than the dry habitat plants.

Habitat and drought effects on morphological traits and biomass accumulation in the drought experiment

Habitat had a significant effect on plant biomass accumulation, resulting in larger total biomass in plants from wet habitats than in plants from dry habitats in both control and drought treatments (Fig. 3a, Table 2). Similar to total plant biomass, root biomass per fraction was also significantly influenced by habitat, with higher root biomass per fraction in plants originating from wet than from dry habitats (Fig. 4, Table 3). This habitat difference in root biomass was enhanced by drought stress (Fig. 4, Table 3). However, habitat did not affect most other morphological traits, such as specific leaf area (SLA) and root to shoot ratio, neither did it effect other root traits of interest, including rooting depth, specific root length (SRL), average root diameter, root surface area (RSA) and root volume per root dry weight (Fig. 3 & 4, Table 2). Moreover, root length percentage per diameter class, which measures relative root length per diameter class, was not affected by habitat, either (Table 4).

Drought significantly inhibited plant growth, as shown by decreased final plant biomass, but drought did not influence the biomass allocation pattern between root and shoot (Fig. 3, Table 2). Root biomass was also negatively affected by drought (Fig. 4, Table 3). Unexpectedly, SLA was remarkably larger under drought stress compared to the control conditions (Fig. 3, Table 2). Moreover, root volume, rooting depth and root biomass were significantly reduced by drought stress (Fig. 3 & 4, Table 2 & 3). However, other root traits,

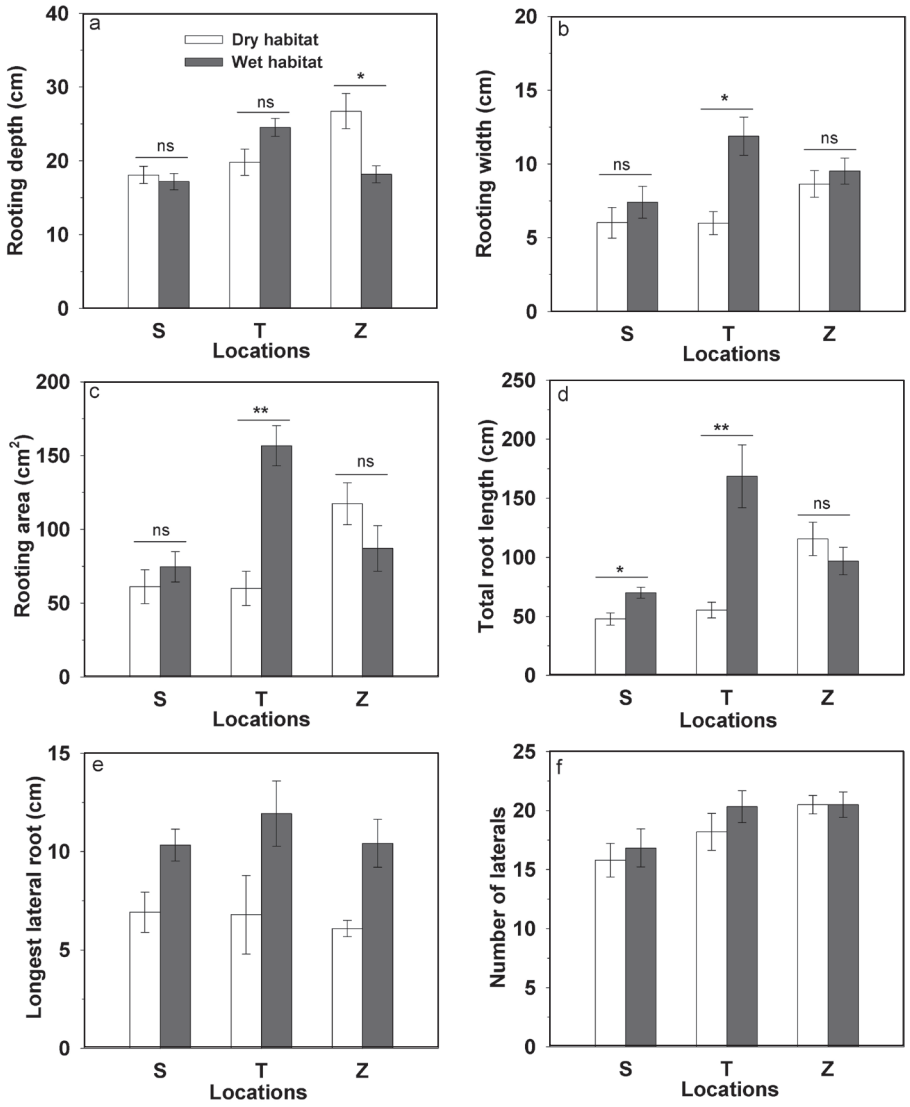

Figure 2. Rooting depth (a) and width (b), rooting area (c), total root length (d), longest lateral root length (e) and number of lateral roots (f) of *S. dulcamara* plants after growing on blue germination paper for two weeks. Seeds were collected from both dry and wet habitats in each of the three locations (S, T and Z). S = Schiermonnikoog, T = Texel, Z = Zandvoort. Habitat effect within each location was analysed for rooting depth, rooting width, rooting area, and total root length. Means ± 1 SE are given; n = 6. The significance levels are indicated as: $^{***}p \leq 0.001$, $^{**}0.001 < p \leq 0.01$, $^{*}0.01 < p \leq 0.05$, $^{\$}0.05 < p \leq 0.1$, $^{ns}p > 0.1$.

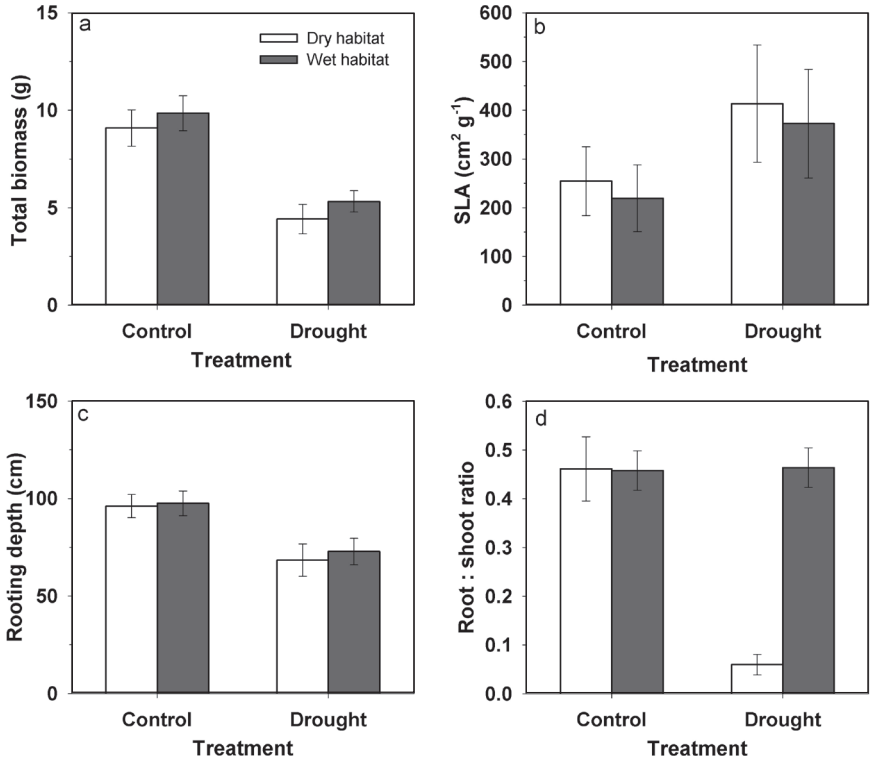

Figure 3. Mean (± 1 SE) of total biomass (a), specific leaf area (SLA; b), rooting depth (c) and root to shoot ratio (d) of *S. dulcamara* plants originating from dry (white bars) and wet (black bars) habitats in control and drought treatments for seven weeks. n = 3.

Table 2 Results of a split-plot ANOVA on total biomass, root to shoot ratio, specific leaf area (SLA), specific root length (SRL), root surface area (RSA) and root volume per root dry weight and average root diameter, with treatment and habitat as fixed factors and habitat nested within location as a random factor. Rooting depth was analysed by means of a split-plot ANOVA, with the same fixed factors and random factor as in the ANOVA model. Significant and marginally significant Chi-squares were in bold; $^{***}p \leq 0.001$, $^{**}0.001 < p \leq 0.01$, $^{*}0.01 < p \leq 0.05$, $^{\$}0.05 < p \leq 0.1$, $^{ns}p > 0.1$.

		Chi-squares							
	d.f.	**Total biomass**	**Root to shoot ratio**	**SLA**	**SRL**	**RSA per DW**	**Root volume per DW**	**Root diameter**	**Rooting depth**
Treatment (T)	1	**159.728*****	2.469^{ns}	**9.597****	0.680^{ns}	2.619^{ns}	**3.871***	1.748^{ns}	**68.829*****
Habitat (H)	1	**4.200***	0.007^{ns}	1.479^{ns}	0.031^{ns}	0.197^{ns}	0.065^{ns}	1.083^{ns}	0.130^{ns}
T × H	1	0.079^{ns}	1.684^{ns}	0.117^{ns}	1.423^{ns}	0.247^{ns}	1.078^{ns}	**6.014***	0.403^{ns}

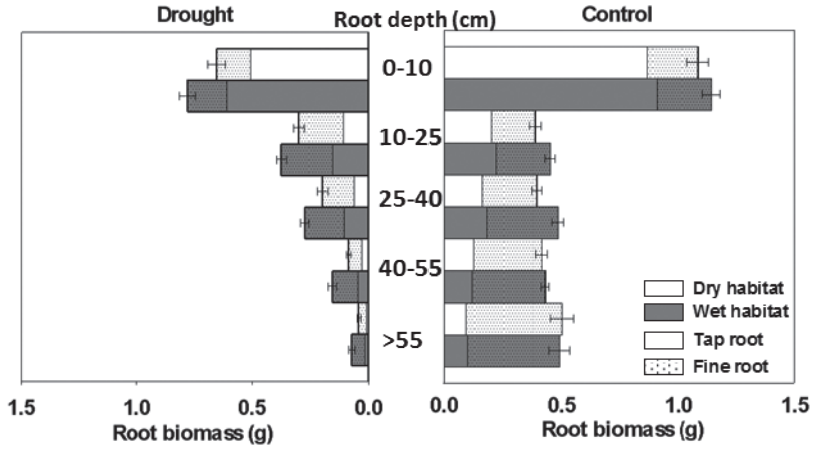

Figure 4. Tap root and fine root biomass per root fraction of *S. dulcamara* plants originating from dry (light bars) and wet (dark bars) habitats in both control (right panel) and drought (left panel) treatments for seven weeks. Means are given, error bars indicate SEs; n = 3.

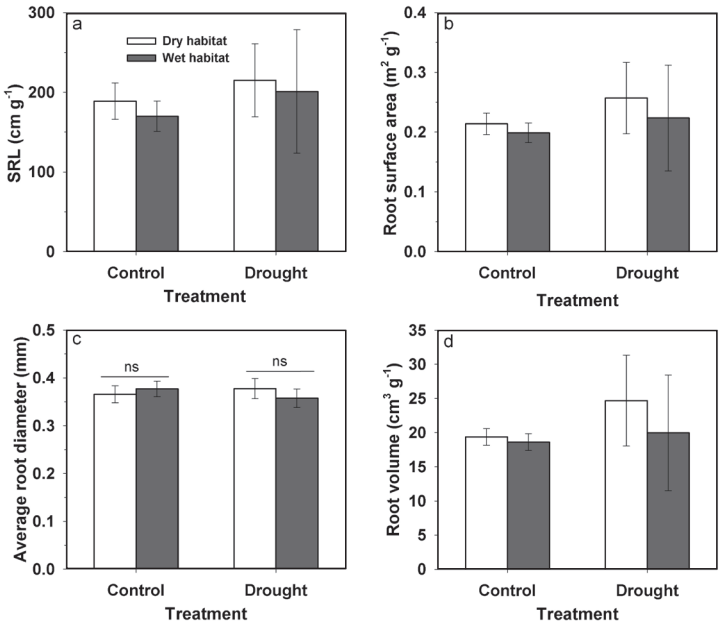

Figure 5. Specific root length (SRL; a), root surface area per root dry weight (b), average root diameter (c) and root volume per root dry weight (d) measured on the fine roots from the third root fraction (25-40 cm) of *S. dulcamara* plants originating from dry (white bars) and wet (black bars) habitats in both control and drought treatments for seven weeks. Means are given, error bars indicate SEs; n = 3. Significance between habitats within each treatment was indicated: ns $p > 0.05$.

Table 3 Results of a three-way split-plot ANOVA on tap root biomass, fine root biomass and total root biomass at each fraction, with treatment, habitat and fraction as fixed factors, habitat nested within location as a random factor. Significant and marginally significant Chi-squares were in bold; $^{***}p \leq 0.001$, $^{**}0.001 < p \leq 0.01$, $^{*}0.01 < p \leq 0.05$, $^{\$}0.05 < p \leq 0.1$, $^{ns}p > 0.1$.

		Chi-squares		
	d.f.	**Root biomass**	**Tap root biomass**	**Fine root biomass**
Treatment (T)	1	**178.128*****	**73.953*****	**13.807*****
Habitat (H)	1	**7.059***	0.016^{ns}	**5.430**$^{\$}$
Fraction (F)	4	**255.544*****	**826.048*****	**21.884*****
T × H	1	**6.209***	1.016^{ns}	2.715^{ns}
T × F	4	**74.918*****	**12.207***	**11.011**$^{\$}$
H × F	4	4.068^{ns}	1.880^{ns}	4.482^{ns}
T × H × F	4	**10.713**$^{\$}$	0.294^{ns}	2.260^{ns}

Table 4 Results of three-way split-plot ANOVA on the percentage of root length at each diameter class, with treatment, habitat and diameter class as fixed factors, habitat nested within location as a random factor. Significant and marginally significant Chi-squares were in bold; p values were adjusted by Bonferroni correction, $^{***}p \leq 0.0003$, $^{**}0.0003 < p \leq 0.0033$, $^{*}0.0033 < p \leq 0.017$, $^{\$}0.017 < p \leq 0.0333$, $^{ns}p > 0.0333$.

	Chi-squares	
	d.f.	**% root length**
Treatment (T)	1	1.173^{ns}
Habitat	1	1.300^{ns}
Diameter	2	**1036.891*****
T × H	1	0.021^{ns}
T × D	2	**7.179***
H × D	2	1.395^{ns}
T × H × D	2	0.612^{ns}

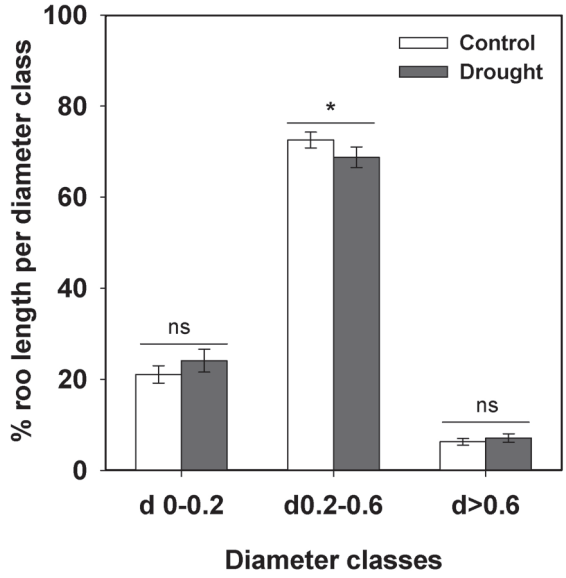

Figure 6. Percentage of root length per root diameter class calculated by dividing root length of a specific diameter class by the total root length of all three diameter classes of *S. dulcamara* plants in both control (white bars) and drought (black bars) treatments for seven weeks. Means are given, error bars indicate SEs; n =6. The significance levels are indicated as: $^{*}0.01 < p \leq 0.05$, $^{ns}p > 0.1$.

such as SRL, RSA and average root diameter were not influenced by drought (Fig. 5, Table 2). Although drought did not influence fine root length, it did alter the root length partitioned to different diameter classes (Fig. 6, Table 4). Drought significantly decreased root length in the diameter class 0.2-0.6 mm, but did not influence the length of thinner (0-0.2 mm) or thicker roots (>0.6 mm) (Fig. 6, Table 4).

Discussion

The root system is the first organ to perceive drought stress. As different rooting patterns may be favoured under different soil moisture conditions, root traits are likely to be under selection, thus resulting in differentiation in root traits among habitats with contrasting soil moisture. Using the plant species *Solanum dulcamara*, which inhabits such contrasting hydrological environments, we investigated whether contrasting habitats of origin lead to differentiation in constitutive rooting patterns and/or differences in root

plasticity in response to drought stress. In contrast to our expectation but in line with previous chapters (e.g. Chapter 2, 3 & 4), we found limited habitat differentiation in constitutive root traits (in plants not subjected to drought stress). Similar to the previous results which were obtained in relatively small pots, even in containers with the potential for deep rooting, the effect of habitat on root growth and on the drought response was limited in this species. This work thus confirms the conclusion drawn in Chapter 2 that flooding and drought did not lead to ecotypic differentiation in *S. dulcamara* occurring in flooding- and drought-prone environments. We will discuss these findings and speculate on the survival strategy of *S. dulcamara* in the dry dunes, where top soil water content can reach as low as 2-3 % during the growing season (Chapter 2).

Limited habitat effects on root traits

Contrasting environmental conditions often lead to divergent selection for adaptive traits (Gianoli & Gonzalez-Teuber 2005). For instance, greater water use efficiency was found in plants from dry sites than from wet sites under drought conditions in both *Impatiens capensis* (Heschel *et al.* 2002) and *Boechera holboellii*, a perennial relative of *Arabidopsis thaliana* (Knight *et al.* 2006). To our surprise, very limited differentiation in constitutive root traits was revealed in populations of *S. dulcamara* from dry and wet habitats. Our results suggest that contrasting local water regimes have not resulted in the selection of distinctive rooting patterns in this species, and in similar drought resistance across a range of habitats. However, habitat of origin did significantly affect the constitutive total root length, which may result in potential differences in water uptake if this differentiation in total root length between habitats is maintained in plants growing under drought stress. Plants from dry habitats also tended to produce shorter lateral roots than those from wet habitats, which may suggest the potential for a stronger allocation to deep roots rather than to laterals as there are trade-offs between tap root elongation and lateral root growth (Lynch 2011). However, these differences did not translate to differences in plants growing in the long tubes. Drought did not induce population differentiation in SLA and any of the root related traits that were investigated; instead, drought induced similar levels of plastic responses in plants originating from both habitats. These results are in agreement with what has been shown in chapter 2, i.e.,*S. dulcamara* does not evolve locally adapted genotypes, but induces similar levels of phenotypic plasticity in plants from both, dry and wet habitats. These findings are not unprecedented. Plants of *Polygonum persicaria* from contrasting moisture regimes showed

similar patterns of plastic responses, i.e., root biomass allocation and water use efficiency, to drought stress (Heschel *et al.* 2004). Such plasticity evidently may contribute to the wide range of hydrological niches of these two species.

Drought effects on plant growth and root plasticity

Drought as an environmental constraint generally impedes plant growth (Bray 1997; Pierik & Testerink 2014). In our study, drought significantly reduced biomass production in plants from both dry and wet habitats. To alleviate the negative effects of drought stress, plants are able to alter their shoot and/or root morphology to reduce water loss and increase water acquisition during drought stress (Chaves, Maroco & Pereira 2003; Comas *et al.* 2013). One of the common acclimations found in drought adapted species is producing small and thick leaves to minimise transpiration area, resulting in decreased SLA in drought-stressed plants (Kooyers *et al.* 2015). However, in the current study, *S. dulcamara* plants originating from both habitat types showed higher SLA in the drought than in the control treatment (Zhang *et al.* 2015). Also contrary to our predictions, *S. dulcamara* did not increase biomass allocation to the root system under drought stress, as apparent from the similar root to shoot ratio compared to control plants. Similarly, rooting depth, another adaptive trait in response to drought stress, was not increased but significantly decreased by drought in the present study. All these changes in the expression of root traits in *S. dulcamara* under drought stress are not in line with other drought-tolerant species (Gowda *et al.* 2011; Comas *et al.* 2013), which would be expected to result in reduced plant fitness under conditions of drought. This suggests that *S. dulcamara* is not a typical drought tolerant species, and drought occurring at very early developmental stage may pose detrimental effects on plant growth and survival. As this species does occur in very dry sand dunes with high chance for severe summer drought, other mechanism may help this species to thrive in such field conditions, and it will be intriguing to unravel them in future work.

Solanum dulcamara is capable of fast root elongation under non-stressed conditions, as shown in the current study, where the roots of *S. dulcamara* reached about 1 m depth within seven weeks under control conditions. Such fast root growth under conditions where water availability is not limited may enable plants to send roots into the deep soil layers during periods of sufficient precipitation, thereby enabling the plants to maintain water uptake from deep soil during subsequent topsoil drought. The fact that young seedlings are not very common in the dry sand dunes (personal observation) may indicate that seedling recruitment is sensitive to drought, and regrowth from root systems that already are rooted in the deep soil might be the primary mechanism for

S. dulcamara to persist in dry sand dunes. Recruitment could rely on exceptionally wet spring and early summer conditions, providing the seedlings with sufficient time to reach deeper soil layers before the onset of drought later in the season.

Conclusions

Consistent with results provided in previous chapters, this study again showed very limited population differentiation in both, constitutive root traits and root plasticity in response to drought, which confirms that *Solanum dulcamara* has not evolved locally adapted genotypes in contrasting hydrological environments. Fast root growth into deep moist soil layers under sufficient water availability rather than the typical drought avoidance trait, i.e., drought-induced deep rooting, may be the main mechanism enabling *S. dulcamara* plants to survive in the dry sand dunes. Plants that have reached deep soil layers with sufficient moisture remaining after wetter seasons may be able to survive subsequent summer drought and contribute to the persistence of this species in the drought-prone environments.

Chapter 7

Synthesis

Plants are continuously subject to changes in the environment. This brings challenges for growth and performance, and forces plants to adapt to these altering conditions throughout their life cycle. Extreme patterns of precipitation, for instance, result in adverse conditions such as flooding or drought stress. Climate change, which is projected to increase the frequency and intensity of flooding and drought events (IPCC 2013), may increase the frequency by which plants are confronted with such extremes in the near future. Nearly all crop species are vulnerable to flooding and drought stress, which prevents studies that investigate plant adaptations in response to flooding and drought in crop species. However, gaining knowledge from wild species that are tolerant to the stress may shed light on the mechanisms enabling plants to survive in a variety of moisture conditions. Eventually, this information may be utilized in developing new breeding strategies that aim at improving flooding and drought tolerance in crop species. In this study, we aimed at unravelling the adaptive phenotypic responses in a wild species, *Solanum dulcamara*, to flooding and drought stress. *S. dulcamara* was selected due to its wide ecological range and its tolerance to both flooding and drought stress. This makes the species an ideal study system to investigate the mechanisms enabling survival in different soil moisture conditions. Below, I will summarize the most important findings of this thesis, followed by main conclusions and speculations on phenotypic plasticity and adventitious root formation.

Main results and conclusions

It is not common for a given plant species to be able to survive contrasting stresses, as such contrasting stresses usually require opposing adaptive traits which may be mutually exclusive. This is reflected by the fact that contrasting habitats are usually inhabited by a distinct set of species, each well adapted to its specific habitat conditions and with a low likelihood to survive and prosper in the contrasting habitat. However, *S. dulcamara* is one of the few species that covers the whole range of soil moisture conditions. To get a better insight into how the species governs this broad distribution, I first investigated the mechanisms allowing *S. dulcamara* to survive both, flooding and drought stress (**Chapter 2**). I estimated the genetic differentiation in plants of nine pairs of populations, of which each pair originated from hydrologically strongly contrasting habitats, using neutral molecular AFLP markers. A significant genetic differentiation was present between habitats within each of the nine locations. This provides a potential for local adaptation to the specific habitat to take place. I subsequently estimated the phenotypic differentiation of each

by investigating whether plants experienced an advantage under local rather than under foreign stress conditions. This was done by subjecting the nine pairs of populations to either well-watered control, flooding, or drought treatments in the greenhouse. In contrast to my expectations, I did not find a consistent difference in the quantitative traits and biomass production between plants originating from the two contrasting habitats, neither under control conditions nor in the responses to flooding and drought stress. This indicates that flooding and drought did not lead to ecotypic differentiation in *S. dulcamara*. Instead, plants regardless of habitat of origin were all characterised by high levels of phenotypic plasticity in their responses to flooding and drought stress, indicating that despite presumably restricted gene flow between habitats, a high level of plasticity rather than local adaptation is the main mechanism enabling *S.dulcamara* to span a wide ecological gradient and to survive under contrasting environmental conditions.

In **Chapter 3**, I investigated whether *S. dulcamara* plants at different developmental stages display different phenotypic responses to shallow and deep flooding. I subjected two out of the nine population pairs to different water depths in order to be able to establish general patterns for flooding responses at different developmental stages. Both juvenile (4-week-old; without preformed adventitious root primordia) and mature (8-week-old; with preformed adventitious root primordia) plants produced adventitious roots during partial flooding. Adventitious root formation, however, was delayed in juvenile plants. Complete submergence suppressed the emergence of adventitious roots in both juvenile and adult plants. If a flooded shoot restored air contact, this suppression was subsequently released, suggesting that the expression of an adaptive response to flooding may depend on flooding type, with intensified flooding stress potentially inhibiting the common adaption. Irrespective of developmental stage, the quantity and biomass of adventitious roots were positively correlated with plant biomass, which potentially implied a positive role of these roots in plant growth during flooding. This hypothesis was further explored in **Chapter 4**. Similar to the results in **Chapter 2,** no difference was found in both adventitious root formation and plant biomass production in plants originating from dry and wet habitats.

The contribution of adventitious roots to plant performance during flooding was then quantified by manipulating the adventitious root formation (**Chapter 4)**. The results clearly showed that the effect of adventitious roots on plant growth depended on flooding duration, as plants benefitted more from the presence of adventitious roots in long-term flooding (> 2 weeks) than in short-term flooding. Despite the relatively short time-lag between the onset of

flooding and the initiation of adventitious roots, the adventitious root system needed time to develop before it was large enough to take over part of the function of the original roots, such as nutrient uptake, and only then increased plant performance. Nutrient uptake was positively correlated with the surface area of adventitious roots, and water uptake as estimated by transpiration rate was also enhanced by the presence of adventitious roots although the increase was not statistically significant. Consequently, as an adaptive trait, adventitious root formation is likely to be selected for in habitats experiencing long-term flooding. In contrast to my initial expectation, adventitious roots did not incur clear costs in terms of biomass reduction during formation and maintenance in short-term flooding. The low costs associated with adventitious root formation may therefore suggest that it is unlikely for this trait to be selected against in rarely flooded environments.

Complete submergence is a compound stress that may induce changes in a variety of environmental factors which in turn may affect the potential of plants to produce adventitious roots. As shown in **Chapter 3**, the adaptive adventitious root formation was constrained in complete submergence. Compared to partial flooding, complete submergence further decreases the availability of multiple resources such as oxygen, carbon dioxide and light, which inevitably causes carbohydrate deficiency as well as leading to reduced transport of essential hormones such as auxin. To obtain a better understanding of these factors that might limit initiation and development of adventitious roots, in **Chapter 5**, I explored the role of external oxygen concentration, stored and newly acquired carbohydrates as well as auxin availability in adventitious root formation. Oxygen, carbohydrates and auxin are all likely to be of importance for this process. Particularly, internal diffusion of oxygen from distal shoot parts played a stronger role in adventitious root development than dissolved oxygen in the floodwater. Similarly, carbohydrates formed during flooding rather than carbohydrates acquired prior to flooding and stored in the plant tissue appeared to be used for adventitious root formation. This suggests that *S. dulcamara* in shaded habitats is unlikely to be equally well adapted to flooding as plants in open areas.

As shallow pots such as used in **Chapter 2** constrain the development of deep roots, the plants may have been limited in their response to drought stress in these experiments**.** Divergent selection may be expected to act on rooting depth under dry, but not under wet conditions. Therefore, a screening on constitutive root phenotypes of all the nine population pairs was conducted in **Chapter 6**. This screening showed limited differentiation in most constitutive rooting traits. To investigate whether the plasticity of rooting traits differed

between habitats, plants from three population pairs were subjected to control and drought treatments in long tubes (1.3 m) that allowed plants to root deep. Consistent with the previous results on root development, habitat led to little differentiation in plastic rooting traits. Plants produced larger and deeper root systems under well-watered than drought conditions regardless of habitat of origin. This suggests that local environmental conditions did not result in differential plasticity in rooting traits, and deep rooting might be constitutively expressed in plants instead of being a drought-induced trait.

With the combined evidence in this thesis, the conclusion can be drawn that evolving contrasting ecotypes each adapted to flooding and drought stress, respectively, is not the primary strategy for *S. dulcamara* enabling it to occupy hydrologically contrasting environments. In contrast, maintaining high levels of plasticity is likely the major factor allowing this species to occur in both flooding- and drought-prone habitats.

Surviving flooding and drought stress: comparisons with other species

Species characterised by a wide ecological niche often produce distinct ecotypes showing specific adaptation to and hence increased performance in their respective local habitats (e.g. Galloway & Fenster 2000; Lenssen *et al.* 2004; Becker *et al.* 2006; Wright, Stanton & Scherson 2006; Banta *et al.* 2007; Grøndahl & Ehlers 2008; Leimu & Fischer 2008; Moore, Moore & Baldwin 2014; Pickles *et al.* 2015). This may be even more common in species inhabiting strongly contrasting habitats, as more divergent environments are expected to exert stronger and more consistent opposing selection forces leading to divergent evolutionary changes (Hereford 2009). Even the compelling environmental differences, flooding and drought, which are the two extremes along a hydrological gradient, did not result in clear adaptive population differentiation in *S. dulcamara* (Chapter 2 & 6). In addition to showing hardly any constitutive differences, populations of this species originating from contrasting habitats displayed similar degrees of phenotypic plasticity in traits related to flooding and drought resistance. This ability to rapidly change the phenotype to match different environments independent of the conditions plants have been exposed to in their natural environment so far, might enable this species to persist under future climate change and assist expansion of its distribution. This has already been shown by the successful establishment and spread of *S. dulcamara* as a non-native species in North America and becoming a species with a circumpolar distribution (Waggy 2009).

Phenotypic plasticity has been shown for other species to provide an important means for surviving both flooding and drought stress. For instance, under conditions where continuous gene flow from the source population to the sink population prevented ecotype formation in local environments, a blueberry species (*Vaccinium elliottii*) was able to express phenotypic plasticity in a suite of traits, enabling this species to colonize and persist in the upland (source) habitat as well as the more stressful bottomland (sink) habitat (Anderson, Sparks & Geber 2010). Similarly, young holm oak (*Quercus ilex*) plants from habitats with different soil moisture (from semiarid to humid) were all able to strongly regulate their water use efficiency and stomatal conductance in response to drought conditions without showing habitat differentiation in the expression of such trait plasticity (Gimeno *et al.* 2009). By contrast, in species with a shorter growing cycle, such as the annual species *Impatiens capensis,* ecotype formation in response to soil moisture conditions has been shown to occur (Heschel et al). Even along a small-scale flooding gradient, *Ranunculus reptans* manages to survive environments with different flooding regimes by evolving locally adapted ecotypes (Lenssen *et al.* 2004). Although the length of life cycle of a species might play a role in the surviving strategies, it is still difficult to conclude why some species show ecotype formation in various environments and others don't.

Similarly, local adaptation, rather than high levels of phenotypic plasticity, seems to be more prominent in agricultural systems, showing that traits related to flooding and drought resistance can be selected upon (Setter & Laureles 1996; Wasson *et al.* 2012; Uga *et al.* 2013). A well-studied system for flooding and drought tolerance in agriculture is rice. Rice is an important crop species that can be grown under a variety of environmental conditions (Sauter 2000). Different cultivars are adapted to each of the local conditions, including rainfed uplands that may experience mild drought and areas that are deeply flooded for several months (Kende, van der Knaap & Cho 1998; Vriezen, Zhou & van der Straeten 2003). For instance, enhanced shoot elongation was reported to be beneficial for deepwater floating rice, but adverse for cultivars that originate from habitats with prevalent flash-floods (Setter & Laureles 1996). Although rice plants do not have high drought resistance, deep rooting expressed in specific upland cultivars does enhance their resistance to drought stress (Uga *et al.* 2013). Comparing rice with *S. dulcamara*, the main difference causing the two species employing different strategies in response to flooding and drought stress might be different levels of selection pressures imposed on these two species. Rice is a crop species, the variation in its adaptive traits is intensified by breeding which exerts much stronger artificial selection

forces than the selection in natural ecosystem. Additionally, differences in herbivory, interspecific competition and scale of resource heterogeneity between these two species may lead to different phenotypic plasticity. As in farmlands, herbivores are often (partly) under control, the management of drainage and/or irrigation systems also reduces fluctuations in water depth and weed management and agricultural practices reduce competition and heterogeneity of resources. All these factors presumably decline the potential for high phenotypic plasticity in agricultural systems. In theory, finding out the mechanism underlying this high phenotypic plasticity would provide valuable insights on creating "smarter" crops that can plastically adapt to both flooding and drought stress. This is relevant to do because climate extremes with alternating drought and flooding events are becoming more prevalent due to global climate change.

Adventitious root formation: its contribution and formation

Wetland plant species commonly produce adventitious roots in response to flooding (Colmer & Flowers 2008). While their formation has long been proposed to be beneficial for plant performance during flooding (Jackson & Drew 1984), the number of empirical studies quantifying the contribution of adventitious roots to plant growth is still limited. In this thesis, I evaluated the potential benefits of adventitious roots of *S. dulcamara* in terms of increased performance of flooded plants, and found that the benefits associated with adventitious roots were only present in plants subjected to long-term flooding, but not if flooding lasted shorter than three weeks (Chapter 4). Although the positive contribution of adventitious roots to plant growth had been previously shown for other species (Tsukahara & Kozlowski 1985; Javier 1987; Rich, Ludwig & Colmer 2012), this is the first time that the contribution of adventitious roots to increased performance was linked to flooding duration. I also showed a positive correlation between nutrient uptake and the size of adventitious roots which is strongly influenced by flooding duration. Therefore, the relationship between the contribution of adventitious roots to plant growth and duration of flooding was likely attributable to the difference in nutrient uptake by these adventitious roots that varied significantly in quantity and morphology in short-term *vs.* long-term flooding (Fig. 3, Chapter 4). Costs associated with the expression of plasticity may potentially counteract selection for plasticity. However, in *S. dulcamara* these costs for producing and maintenance of the adventitious roots appear to be negligible (Chapter 4). Plants from flooding-

prone environments are therefore likely to carry this trait regardless of the prevalent duration of flooding. These data, though, do not show whether the ability to produce adventitious roots is associated with costs. This would require the comparison of genotypes or mutants lacking the ability to initiate adventitious roots under flooded conditions. However, the fact that all plants, irrespective of the habitat of origin, produce adventitious roots indicates that potential costs associated with the maintenance of this trait are not likely to be significant.

With regard to the apparent benefits, adventitious roots are expected to be induced in plants under flooding stress. However, the formation of these roots was influenced by multiple resources, lack or inadequate supply of some of which may impede this process. The duration until the onset of adventitious root emergence is species-specific, ranging from a few hours to several days upon flooding (Steffens & Rasmussen 2016). The formation of adventitious roots in *S. dulcamara* was relatively quick, and took 2-3 d under partial flooding (Chapter 3; Dawood *et al.* 2014). This is similar to tomato, sunflower and *Rumex palustris* (Wample & Reid 1978; Visser, Blom & Voesenek 1996; Vidoz *et al.* 2010), but somewhat slower than in rice plants (Lorbiecke & Sauter 1999). Within a single species, the developmental stage of the plant also significantly influences the time until adventitious root emergence. Juvenile *S. dulcamara* plants (without preformed adventitious root primordia) had a delay in adventitious root formation of about 20 d and produced fewer adventitious roots than adult plants that exhibited preformed adventitious root primordia prior to flooding (Chapter 3). Such delay is probably due to the lack of preformed adventitious root primordia. Similar to *S. dulcamara*, the timing of adventitious root emergence also showed a dependence on the age and developmental stage of the respective node in rice (Lorbiecke & Sauter 1999; Steffens *et al.* 2012), which is probably due to difference in the number of parenchymal cell layers that cover on the adventitious root primordia on young and old nodes.

Another well studied example for adventitious root formation is in plant cuttings. There are similarities and also differences in the effects of different factors on the flooding-induced adventitious root formation and the formation of new roots in plant cuttings. In pea cuttings, the effects of developmental stage on adventitious root formation seemed to be more complicated than in *S. dulcamara* (Rasmussen *et al.* 2015). For instance, when making cuttings from a shoot within the vegetative phase in pea plants, older shoots produced significantly larger number of adventitious roots, however, when making cuttings from a shoot in flowering phase, adventitious root production of these

cuttings significantly decreased with the increase in shoot age (Rasmussen *et al.* 2015). The depletion of carbohydrates prior to flooding did not exert a significant influence on the adventitious root formation in *S. dulcamara*, which is opposite to the previously shown effect of carbohydrate depletion on adventitious root formation in petunia and carnation cuttings (Klopotek *et al.* 2010; Agulló-Antón *et al.* 2011). Carbohydrate depletion through a dark exposure resulted in faster formation and larger quantity and size of adventitious roots in both petunia and carnation cuttings (Klopotek *et al.* 2010; Agulló-Antón *et al.* 2011). This positive effect of carbohydrate depletion on adventitious root formation in cuttings was likely due to a sink establishment of carbohydrates by the pre-depletion (Klopotek *et al.* 2010; Agulló-Antón *et al.* 2011), which is not clearly shown in *S. dulcamara*. Similar to results found for plant cuttings of carnation, auxin application substantially stimulated adventitious root formation in *S. dulcamara*. In carnation cuttings, auxin was shown to accumulate sugars in the basal stem, thus stimulating root formation (Agulló-Antón *et al.* 2011). Whether this is also the case in flooded *S. dulcamara* plants needs further investigation.

Concluding remarks

In this thesis, I showed that, as a species occurring over a wide ecological gradient, *Solanum dulcamara* is characterized with high levels of phenotypic plasticity. Low costs of such plasticity in adaptive traits, e.g., adventitious root formation, might be the primary mechanism that enables this species to thrive in environments with prevalent flooding events. Although fast root elongation towards deep moist soil layers before top soil desiccates might benefit plant growth during subsequent drought conditions, the clear strategy for this species to survive top soil drought remains unrevealed. Future studies are needed to investigate whether allocating roots in deep soil before drought events commence is indeed the primary mechanism for *S. dulcamara* to survive in the dry dune areas.

From my work it remains surprising and intriguing that the genetically differentiated populations with contrasting habitat of origins did not differentiate in most phenotypic traits, such as adventitious root formation in response to flooding, constitutive root architecture and drought related root traits. The populations do seem to differ in plasticity in flowering time. This difference in phenology in response to flooding and drought may well result in genetic differentiation between the two habitat types. To confirm this hypothesis, further field investigations monitoring plant (inter) annual

phenology and the flooding and drought regimes in both habitats are required. In addition, although flooding and drought stress seem to be prominent selection forces in these two habitat types, other biotic and/or abiotic factors that are likely to lead to divergent selection should not be excluded. Therefore, reciprocal transplant experiments combined with common garden experiments that assessing plant response to each of the different stress factors in their habitats are likely to provide a clearer explanation for the genetic differentiation found between the two habitat types.

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Samenvatting / Dutch Summary

S

Planten zijn continu onderhevig aan veranderingen in hun milieu. Dit brengt uitdagingen met zich mee wat betreft groei en prestaties, en dwingt planten zich gedurende hun levenscyclus aan te passen aan dergelijke veranderingen in hun omgeving. Extremen in neerslag resulteren bijvoorbeeld in ongunstige condities zoals overstromings- of droogtestress. Door klimaatverandering neemt de frequentie en intensiteit van overstromingen en droge periodes naar verwachting toe, en daarmee ook de frequentie waarmee planten in de nabije toekomst worden blootgesteld aan dergelijke extremen. Bijna alle landbouwgewassen zijn gevoelig voor overstroming en droogtestress. Dit beperkt de mogelijkheden van onderzoek naar adaptaties van planten aan overstroming en droogte in landbouwgewassen. Kennis over adaptaties van wilde soorten die wél tolerant zijn voor deze stressoren kan echter licht werpen op de mechanismen die het voor planten mogelijk maken te overleven in zowel natte als droge condities. Deze informatie kan uiteindelijk gebruikt worden in de ontwikkeling van nieuwe veredelingsstrategieën die erop gericht zijn de overstromings- en droogte tolerantie van gewassen te verbeteren. Deze studie heeft als doel de adaptieve, fenotypische responsen van de wilde soort *Solanum dulcamara* op overstroming en droogtestress te ontrafelen. *S. dulcamara* werd hiervoor gekozen vanwege het brede ecologische verspreidingsgebied en de tolerantie van deze soort voor zowel overstroming als droogtestress. Dit maakt deze soort een ideaal onderzoeksmodel voor het bestuderen van de mechanismen die het overleven in bodems met een grote variatie in vochtgehaltes mogelijk maakt. Hieronder zal ik de belangrijkste bevindingen van dit proefschrift samenvatten, gevolgd door de belangrijkste conclusies en enkele speculaties over fenotypische plasticiteit en adventieve wortelvorming.

Belangrijkste resultaten en conclusies

Het is niet gebruikelijk voor een plantensoort om sterk contrasterende stressoren te kunnen overleven, aangezien deze contrasterende stressoren vaak tegengestelde adaptieve responsen vereisen die elkaar uit kunnen sluiten. Dit wordt gereflecteerd in het feit dat contrasterende habitats vaak bewoond worden door een verscheidenheid aan soorten, elk aangepast aan zijn eigen, specifieke habitatcondities, met een daaruit volgende minder hoge kans op overleving en succes in een contrasterende habitat. *S. dulcamara* is echter een van de schaarse soorten die in de hele range aan bodemvochtgehaltes, van extreem droge tot overstroomde bodems, kan overleven. Om beter inzicht te krijgen in hoe deze soort zich in deze brede range handhaaft, heb ik eerst de mechanismen bestudeerd die het voor *S. dulcamara* mogelijk

maakt te overleven in zowel overstroming als droogtestress (**Hoofdstuk 2**). Ik bepaalde de genetische differentiatie met behulp van neutrale moleculaire AFLP markers in planten van negen populatieparen, waarbij elk paar afkomstig was uit hydrologisch sterk contrasterende habitats. Er bleek een significante genetische differentiatie aanwezig tussen de twee habitats binnen elk van de negen locaties. Dit kan duiden op lokale adaptatie van de populaties voor de specifieke habitats. Hierna heb ik de fenotypische differentiatie van elk paar bepaald door te bestuderen of de planten voordeel ondervinden van de condities in hun eigen habitat tegenover die in de contrasterende habitat. Dit werd gedaan door de negen populatieparen te onderwerpen aan goed-bewaterde controlecondities, overstroming, of een droogtebehandeling in de kas. In tegenstelling tot mijn verwachtingen vond ik geen consistent verschil in de kwantitatieve eigenschappen en biomassaproductie tussen planten afkomstig van de twee contrasterende habitats, noch onder controle condities, noch in respons op overstroming en droogtestress. Dit toont aan dat overstroming en droogte niet geleid hebben tot ecotypische differentiatie van *S. dulcamara*. In plaats daarvan werden alle planten, onafhankelijk van de habitat van herkomst, gekarakteriseerd door een hoge fenotypische plasticiteit in hun respons op overstroming en droogtestress, wat aantoont dat ondanks de vermoedelijk beperkte gene flow tussen habitats, een hoge mate van plasticiteit, meer dan lokale adaptatie, het belangrijkste mechanisme is dat *S. dulcamara* in staat stelt om in een brede, ecologische gradiënt voor te komen en te overleven onder contrasterende milieus.

In **Hoofdstuk 3** onderzocht ik of *S. dulcamara* planten van verschillende ontwikkelingsstadia verschillen in fenotypische responsen gedurende ondiepe en diepe overstroming. Ik onderwierp twee van de negen populatieparen aan verschillende waterdieptes om algemene patronen in overstromingsresponsen in verschillende ontwikkelingsstadia nader te bepalen. Zowel juveniele planten (4 weken oud; zonder voorgevormde adventieve wortelprimordia) als adulte planten (8 weken oud; met voorgevormde adventieve wortelprimordia) produceerden adventieve wortels tijdens gedeeltelijke overstroming. Adventieve wortelvorming was echter vertraagd in juveniele planten. Volledige overstroming onderdrukte de vorming van adventieve wortels in zowel juveniele als adulte planten. Wanneer een overstroomde spruit door groei onder water het contact met de lucht herstelde, werd deze onderdrukking opgeheven en vormden zich adventieve wortels, wat suggereert dat de expressie van een adaptieve respons op overstroming afhankelijk is van het overstromingstype, en waarbij een intensivering van de overstromingsstress de gebruikelijke

adaptatie remt. De kwantiteit en biomassa van adventieve wortels waren, onafhankelijk van het ontwikkelingsstadium, positief gecorreleerd met de biomassa van de plant, wat een positieve rol van deze wortels in plantengroei gedurende overstroming impliceert. Deze hypothese werd verder uitgediept in **Hoofdstuk 4**. Vergelijkbaar met de resultaten in **Hoofdstuk 2** werden hier geen verschillen in zowel adventieve wortelvorming als plant biomassa productie tussen planten van droge en natte habitats gevonden.

De bijdrage van adventieve wortels aan het functioneren van de plant gedurende overstroming werd vervolgens gekwantificeerd door manipulatie van de vorming van adventieve wortels (**Hoofdstuk 4**). De resultaten laten duidelijk zien dat het effect van adventieve wortels op de groei van de plant afhankelijk is van de overstromingsduur, aangezien planten meer voordeel opdeden van de aanwezigheid van adventieve wortels gedurende langduriger overstroming (>2 weken) dan in kortdurende overstroming. Ondanks het relatief korte tijdsverschil tussen de start van overstroming en de eerste initiatie van adventieve wortels had het adventieve wortelsysteem tijd nodig om te ontwikkelen voordat het groot genoeg was om een gedeelte van de functies (bijvoorbeeld nutriëntenopname) van het originele wortelsysteem over te nemen. Alleen dan verbeterden de prestaties van de plant. Nutriëntenopname was positief gecorreleerd met het oppervlakte van de adventieve wortels, en wateropname, geschat op basis van verdampingssnelheid door de plant, was ook verhoogd door de aanwezigheid van adventieve wortels, hoewel deze verhoging niet statistisch significant was. Adventieve wortelvorming als adaptieve eigenschap zal daarom waarschijnlijk vooral geselecteerd worden in habitats waarin de planten gedurende langere tijd gedeeltelijk overstroomd worden. In tegenstelling tot mijn aanvankelijke verwachtingen, had de vorming van adventieve wortels geen duidelijke kosten tot gevolg, zoals verminderde groei gedurende wortelvorming tijdens kortdurende overstroming. De lage kosten geassocieerd met adventieve wortelvorming suggereren daarom dat het onwaarschijnlijk is dat een sterke selectie tegen deze eigenschap plaatsvindt in gebieden die maar zelden overstromen.

Volledige overstroming is een samengestelde stress die veranderingen in verschillende milieufactoren veroorzaakt, en op die manier de potentie van planten om adventieve wortels te vormen kan beïnvloeden. Zoals aangetoond in **Hoofdstuk 3** werd de adventieve wortelvorming belemmerd gedurende volledige overstroming. In vergelijking met gedeeltelijke overstroming neemt de beschikbaarheid van verscheidene hulpbronnen zoals

zuurstof, koolstofdioxide en licht, verder af bij volledige overstroming, wat onvermijdelijk leidt tot zowel koolstof- en zuurstofdeficiëntie als ook een verlaagd transport van essentiële hormonen zoals auxine. Om meer inzicht te krijgen in deze factoren die de initiatie en ontwikkeling van adventieve wortels kunnen belemmeren, onderzocht ik in **Hoofdstuk 5** de rol van de externe zuurstofconcentratie, van opgeslagen en nieuw verworven koolhydraten, alsmede die van auxinebeschikbaarheid in adventieve wortelvorming. Zuurstof, koolhydraten en auxine zijn alle drie vermoedelijk belangrijke spelers in dit proces. Met name de interne diffusie van zuurstof vanuit de distale delen van de spruit speelde een belangrijkere rol in de adventieve wortelvorming dan het opgeloste zuurstof in het overstromingswater. Bovendien bleken vooral de koolhydraten die gevormd werden gedurende de overstroming, meer dan de koolhydraten verworven voorafgaand aan overstroming en opgeslagen in het plantenweefsel, gebruikt te worden voor adventieve wortelvorming. Dit suggereert dat *S. dulcamara* in schaduwrijke habitats waarschijnlijk minder goed aangepast is aan overstroming dan wanneer de planten in een meer open habitat staan.

Aangezien ondiepe potten, zoals gebruikt in **Hoofdstuk 2,** de ontwikkeling van diepe wortels beperken, zou deze manier van opkweek van de planten hun respons op droogtestress kunnen beperken. Een selectie op grote bewortelingsdiepte kan vooral worden verwacht in droge habitats , en juist niet onder natte condities. Een screening naar constitutieve wortelfenotypes van alle negen populatieparen werd daarom uitgevoerd in **Hoofdstuk 6**. Deze screening toonde beperkte verschillen in de meeste constitutieve worteleigenschappen. Om te onderzoeken of de plasticiteit van worteleigenschappen verschilde tussen habitats, werden planten van drie populatieparen onderworpen aan controle- en droogtebehandelingen in lange buizen (1.3 m) die het voor de planten mogelijk maakten diep te wortelen. Vergelijkbaar met de voorgaande resultaten van de wortelontwikkeling, vertoonden planten uit verschillende habitats weinig verschillen in plastische worteleigenschappen. Onafhankelijk van de habitat van herkomst produceerden planten langere en diepere wortelsystemen onder goed-bewaterde condities dan onder droge. Dit suggereert dat lokale milieucondities niet resulteren in een afwijkende plasticiteit van worteleigenschappen, en dat diepe doorworteling constitutief tot expressie komt in planten in plaats van te worden geïnduceerd tijdens droogte.

Uit dit proefschrift kan door combinatie van de diverse resultaten geconcludeerd worden dat het vormen van contrasterende ecotypes die elk aangepast aan respectievelijk overstroming en droogtestress, voor *S. dulcamara* niet de primaire strategie is waardoor deze soort zich in hydrologisch contrasterende milieus kan vestigen. Daarentegen is het in stand houden van een hoge plasticiteit vermoedelijk de voornaamste factor die het deze soort in staat stelt in zowel overstromings- als droogtegevoelige habitats voor te komen.

Acknowledgements

A

Writing this part means my four-year PhD life is coming to the end. Looking back to the very beginning of my PhD makes me realize how fast four years can pass and how lucky I have been during this period of time. I would like to acknowledge numerous people for their inputs to my PhD over the last four years.

The first and foremost gratitude has to go to my promotor Hans de Kroon and co-promotors Eric Visser and Heidi Huber who gave me countless support during the last four years. I feel I could not have had better supervisors. Thank you very much for your guidance, support and encouragement, and I'm particularly grateful for your hard work on reading and correcting my thesis in a very short time by making weekends into work days to let me be able to meet the deadline. Hans, first thank you for taking me to your group. I like this group and am very happy to stay longer. Thank you for trying hard to plan the regular meetings with me in your busy calendar. You sometimes have very different ideas than Eric and Heidi, some of which I found very challenging to deal with, but I do appreciate the different perspectives from you. Thank you so much for telling me "Qian, you have my support" when I told you that I would like to have the defence earlier, and I could not appreciate more for that. Eric and Heidi, you are not only supervisors to me, you are also my mentors and friends. I'm very lucky to have you as my daily supervisors. I appreciate the weekly meetings we had in the first two years. I was completely lost in the first year and didn't know where I should head for. Those meetings were very helpful in guiding me to the right direction. You always have your doors open to me, giving me support and encouragement. Every time I see you, I know that I will be fine because you always have my back. Eric, thank you for making me the digital birthday card for my first birthday in the Netherlands. Your encouraging smile is one of the best things I get from you over the last four years, I'm so afraid that I won't see that smile if I let you down. Heidi, thank you for caring about me like a friend, I really like the conversations we had about anything, such as those about old friends, different cultures, being non-dutch in the Netherlands, family, etc. I will miss you both a lot if I was about to leave soon. I also greatly acknowledge CSC for offering me the grant to support my PhD study in the Netherlands.

I would like to further express my gratitude to our secretaries Jose B., Jose A. and Peter Charpentier for their kind help during those four years. Special thanks go to Jose B., thank you for getting my first room in Nijmegen. What I appreciate most is that you not only got me a room to stay, but also made it a home for me: you even made my bed before I arrived! I like the flower you bought for me. The instant noodles, fruits, energy bars and juice you bought filled my stomach and also warmed my heart! Thank you for arranging the

insurance, rent contract and other documents for me. I always like to go to you and talk to you when I'm not very busy. I'm very happy that you decided not to retire that early.

There is another group of people whom I would like to give many many thanks to, because without their help I wouldn't have finished this thesis. Thank all the technicians in the department Hannie, Annemiek, Peter Cruijsen, Jan Willem and Niels. I deeply appreciate your help, your hard work and technical support to my research. Thank you all for being so nice to me! Hannie, thank you for inviting me to your home in Roermond, I really enjoyed having lunch and dinner in your yard and the small picnic in your land, that was a very special and wonderful day for me! Thank Susan and Wim for their hospitality too. Annemiek, thank you for rescheduling your working days to be able to attend my defence.

Dear (current and previous) PhD fellows and postdocs in Ecology and Molecular Plant Physiology, Marloes, Janneke, Sarah Faye, Maartje, Marco, Casper, Anne, Marjolein, Nils, Dina, Natan, Eva, Marlous, Valerie, Yingying, Ralph, Annieke, Ralf, Ernandes, Onno, Duy, Isabela, Nicky, Emma (Xinping), Jiemeng, Hanjing, Stuart, Nyncke, Monique van Kempen, Christian, Jeroen, Nathan and Raquel, thank you for all the fun during coffee and lunch breaks, day-outs, games, borrels, pizza times, BBQs, sports, etc. You make the department a nice place to work in. Marloes, Janneke and Maartje, thanks a lot for letting your students to help with my first experiment. Marloes, thank you very much for encouraging me when I couldn't find my way at the beginning of my PhD by telling me that "everyone may experience the same at the beginning of his/her PhD", which means a lot to me. Janneke and Maartje, thank you for showing us around in UvA, I feel exactly the same way for my previous university as you do for UvA. Maartje, thank you also for discussing the Qst calculations with me and other help with R. Marjolein and Marco, thank you very much for taking me to the national park and for inviting me for dinner. I liked the interesting indoor-BBQ at Marjolein's and the delicious lasagne made by Marco very much. I deeply appreciate your kindness for trying to remind people to speak English when I'm around! Marco, thank you for being a very nice officemate, too. Annieke, thank you for teaching me how to bake a lemon pie and make cookies, and for cooking very tasty curry couscous for me at your place. Thank you and Michael for offering me a free ride to the NAEM meeting. Sarah Faye, Natan, Yingying and Ralf, thank you for the fun Monopoly night and those interesting conversations. Nils, thank you very much for sharing your progress in finishing your thesis and giving me encouragement for finishing mine. Natan, thank you for correcting my English pronunciation, making a tall

snowman with me and helping me with R problems. Natan, Ralph, Dina and Annelien, thank you a lot for teaching me how to skate, that was a special "first time" to me and I had so much fun that night! Dina, you are the one that I should thank the most. Thank you for making the snowman together with me and Natan, which is the first snowman I made in the Netherlands. I also experienced camping for the first time in my life together with you in your tent. I owe my sincere gratitude to you for translating my Dutch summary and being my paranymph. Christian, thank you for your great enthusiasm in research which positively affects the way I see my own research. Onno, thank you so much for all the discussions on Bittersweet and statistics, thank you for being such a nice and interesting person whom I always like to talk to. Duy and Nicky, thank you for inviting me to your group social activities, I enjoyed the bowling, the pizza and the BBQ! Duy, your discussions, suggestions and opinions on Bittersweet are appreciated, too. Yingying, Hanjing, Emma and Jiemeng, thank you for your company and generous help, without having you being around my PhD life wouldn't have been so joyful and easy.

I owe my thanks to my students Diana, Jannah, Daniek, Sander and Monique van Vegchel. Thank you for your excellent contribution to my PhD project, most of which is included in this thesis. Thank you for the fun you brought me during your internships. I would also like to acknowledge other students who helped me when I needed a hand: Ilse, Martine, Simone, Lianne, Fini, Saskia, Tamara, Bart, Kai, Leonie and Annelien, thank you for helping me out! Annelien, also thank you for the interesting conversations on the way to different conferences and in the greenhouse. Nick, thank you for cycling together with me to the triathlon activity. Thank all the students in the departments for talking to me and smiling at me.

Next, I would like to thank people in the other departments and institutions. Germa, Roy and Paul, thank you for answering my technical questions and helping me to find the right tools. Leon, thank you very much for buying me the mug with the Dutch royal family. Dries, thank you for helping me with installing the printer driver. Jan, thank you for recommending good Chinese restaurants in Nijmegen. Janny, thank you very much for carefully reading our paper and I also appreciate your kind encouragement. Ivo, thank you very much for discussing the abnormal experimental results when Eric was not in the department. I owe my sincere gratitude to you and Eric, Heidi and Janny for offering me the job for the grafting project. Titti, thank you for always being interested in the results of my experiments, your questions and suggestions in the labmeetings and Bsweet meetings make me think further and deeper. Nicole, thank you for being the chairman of my thesis committee and also for

your brilliant ideas on Bittersweet. Elke, Wilco, Sarian, Tjisse, Peter de Groot, Else, and Mieke, although we didn't have much communication, thank you for being friendly to me. I would also like to thank you Dr. Philippine Vergeer and Dr. Rafael Rubio de Casas for your suggestions on the Qst-Fst comparison. Qiaoli, thank you for sharing your flooding research with me in the NAEM meetings. Juan, thank you for being friendly and inviting me to your defence.

Now I move from the Huygens building to the greenhouse. Gerard, I'm very grateful to all the help you provided during my PhD. Thank you so much for arranging help and making place for my experiments, without your generous input my experiments wouldn't have gone so smoothly. I definitely benefit a lot from your smart ideas on practical things, too. Your help is very much appreciated. Yvette, thank you for being so friendly and helpful, you made my greenhouse working days much more interesting. Thanks a lot for caring about me and Bowen! Walter, I also appreciate your help a lot, thank you for being interested in my experiments, I had a lot of fun talking to you. Harry, you are a super handy hero, thank you for closing the holes so well in the black containers that I used for flooding experiments. And the way you hung the lamp on top of the plants was amazing, thank you so much for saving me a lot of trouble!

My dear friends both in the Netherlands and in China, I'm lucky to have you in my life. Xuyan, Junxiao, Jie, Jia, Weibin, Jiankang and Jiemeng, you made my stay in a foreign country less "scary" in the first year, I appreciate your comfortable company which reduces my homesickness. We had so much fun together which I miss a lot, thank you for staying together with me on my birthday and at those important festivals. Xiaoqing, thank you for being my first friend in the Netherlands and my paranymph, sharing my life with you is such a nice thing to do. I would like to thank everyone in CALN, you made my life in the Netherlands even colourful. Yanping, Jinyan, Xi, Qing, Qi, Zhige, Lan, Jiao and Cong, thank you for always supporting me no matter where I am. Jinyan and Qing, I missed the most important moments in your life; Xi, I wasn't able to be there to support you when you needed it, but none of you blamed me, thank you for being my friends! Wei and Lin, we started our friendship since we were in Fei-Hai's group, thank you for always understanding me, encouraging me and helping me. A simple "thank you" would not express my gratitude. My previous supervisors Fei-Hai and Ming-Xiang, Hong-Li and Fangli in Fei-Hai's group, thank you so much for caring about my progress and giving me good advice on my career even I'm not in your group anymore, and for giving me a warm welcome every time I went back to Beijing. Special thanks to Fei-Hai for helping me with finding a future job in China. Zhenming, thank you very much for being a good mentor and friend! Bicheng, thank you for sharing your ideas

on research, R and future career with me. Ruihua, thank you for having me when I was back in Beijing.

Last but not the least, I would like to thank my boyfriend and my family. Bowen, thank you for helping me with leaf scanning and watering plants in the greenhouse during weekend. Thank you for cheering me up when I'm down, you are one of the best things I get in my life, thank you for your love and support. My deepest gratitude goes to my beloved parents and my younger brother. 亲爱的爸爸妈妈，你们是我最应该感谢的人，谢谢你们一直以来对我的疼爱，也感谢你们放手让我去选择自己的人生，永远在背后支持我，我会一直努力。亲爱的弟弟，你在我离开的这几年长大了很多，我们变得越来越有话说了，我真的很高兴你的成长，爸爸妈妈有你在我放心，谢谢你替我照顾他们，这本论文里有你很大的功劳！

CV & Publications

CV

Qian Zhang (张倩) was born on November 15, 1986 in Hebei province, China. In 2006, she started her four-year bachelor study in Beijing Forestry University, during which she developed strong interest in plants and their environment. Therefore, after obtaining her bachelor degree in Soil and Water Conservation in 2010, she continued her study as a master student in the direction of Wetland Plant Ecology under the supervision of Prof. dr. Feihai Yu and Prof. dr. Mingxiang Zhang. During her master study, she worked on the effects of different abiotic and biotic factors, e.g. light, disturbance, and water depth and fluctuation, on the composition and structure of submerged macrophyte communities. In 2012, with the support from the China Scholarship Council she came to the Netherlands for her PhD study. She conducted her PhD research in the Department of Experimental Plant Ecology, Radboud University Nijmegen, under the supervision of Dr. Eric Visser, Dr. Heidrun Huber and Prof. dr. Hans de Kroon. She focused on the responses of *Solanum dulcamara* to flooding and drought stress. The results of her research are described in this thesis. In Octorber 2016, she will start her postdoc research on identifying candidate rootstocks for tomato to improve its stress tolerance, e.g., flooding, drought and salt tolerance in the Departments of Molecular Plant Physiology and Experimental Plant Ecology, supervised by Dr. Ivo Rieu, Dr. Eric Visser, Dr. Heidrun Huber and Dr. Janny Peters.

List of Publications

Manuscripts and publications

1. **Zhang Q,** Huber H, Boerakker J, Bosch D, van Vegchel M , Yang X-P, Rieu I, de Kroon H, Visser EJW. 2016. Factors affecting adventitious root formation during complete submergence of *Solanum dulcamara*. (manuscript in preparation)
2. **Zhang Q**, Huber H, Beljaars S, Birnbaum D, de Best S, de Kroon H, Visser EJW**. 2016.** The beneficial role of newly developed adventitious roots depends on flooding duration: linking plant performance to root functioning. (manuscript in preparation *for Annals of Botany*)
3. **Zhang Q**, Peters JL, Visser EJW, de Kroon H, Huber H. **2016**. Hydrologically constrasting environments induce genetic but no phenotypic differentiation of *Solanum dulcamara*. ***Journal of Ecology***, doi: 10.1111/1365-2745.12648. (in press)
4. Wang P*, **Zhang Q* (* co-first authors),** Xu Y-S, Yu F-H. **2016**. Effects of water level fluctuation on the growth of submerged macrophyte communities. ***Flora***, doi: 10.1016/j.flora.2016.05.005. (in press)
5. **Zhang Q**, Visser EJW, de Kroon H, Huber H. **2015**. Life cycle stage and water depth affect flooding-induced adventitious root formation in the terrestrial species *Solanum dulcamara*. ***Annals of Botany***, 116: 279-290.
6. Dong B-C, Alpert P, **Zhang Q**, Yu F-H. **2015**. Clonal integration in homogeneous environments increases performance of *Alternanthera philoxeroides*. ***Oecologia***, 179: 393-403.
7. Li H-L, Wang Y-Y, **Zhang Q**, Wang P, Zhang M-X, Yu F-H. **2015**. Vegetative propagule pressure and water depth affect biomass and evenness of submerged macrophyte communities. ***PloS ONE***, 10: e0142586.
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9. **Zhang Q**, Xu Y-S, Huang L, Xue W, Sun G-Q, Zhang M-X, Yu F-H. **2014**. Does mechanical disturbance affect the performance and species composition of submerged macrophyte communities? ***Scientific Reports***, 4.
10. **Zhang Q,** Dong B-C, Li H-L, Liu R-H, Luo F-L, Zhang M-X, Lei G-C, Yu F-H. **2012**. Does light heterogeneity affect structure and biomass of submerged macrophyte communities. ***Botanical Studies***, 53: 377-385.

11. Liu R-H, Dong B-C, Li H-L, **Zhang Q**, Yu F-H. **2012**. Patchy distributions of *Spirogyra arcta* do not affect growth of the submerged macrophyte *Ceratophyllum demersum*. ***Plant Species Biology***, doi: 10.1111/j.1442-1984.2011.00346.x.

12. Dong B-C, Liu R-H, **Zhang Q**, Li H-L, Zhang M-X, Lei G-C, Yu F-H. **2011**. Burial depth and stolon internode length independently affect survival of small clonal fragments. ***PloS One***, 6: e23942.

13. Zhang Z-M, Yu X-X, Song S-M, **Zhang Q**, Wu H-L. **2011**. Development of height-diameter models in Beijing mountain area of China. ***VEGETOS***, 24:18-28.

14. Zhang Z-M, Song S-M, Wu H-L, Yu X-X, **Zhang Q**. **2011**. Factors affecting soil erosion in Beijing mountain forestlands. ***African Journal of Biotechnology***, 10: 17134-17141.

Presentations

Oral:

1. Adaptive adventitious root formation allows plants to cope with flooding. 09.02.2016, the Netherlands Annual Ecology Meeting, Lunteren, the Netherlands.
2. Is local adaptation the underlying mechanism enabling *Solanum dulcamara* to inhabit contrasting habitats? 04.14.2015, Experimental Plant Science in the Netherlands, Lunteren, the Netherlands.
3. Adventitious roots as an adaptation to flooding. 29.10.2014, Radboud university IWWR day, Nijmegen, the Netherlands.
4. Bittersweet in response to flooding: developmental stage matters. 29.11.2013, EPS PhD day, Leiden university, the Netherlands.

Poster:

1. Benefits of adventitious roots depend on duration of submergence**:** linking plant performance to root functioning. 24-30.06.2016, Plant Biology Europe EPSO/FESPB 2016 Congress, Prague, the Czech Republic.
2. Phenotypic plasticity rather than local adaptation allows bittersweet to colonize contrasting habitats. 1-4.07.2015, the European Plant Science PhD retreat, Amsterdam, the Netherlands.
3. Response of Bittersweet to flooding: developmental stage matters, 11-12.02.2014, the Netherlands Annual Ecology Meeting, Lunteren, the Netherlands.